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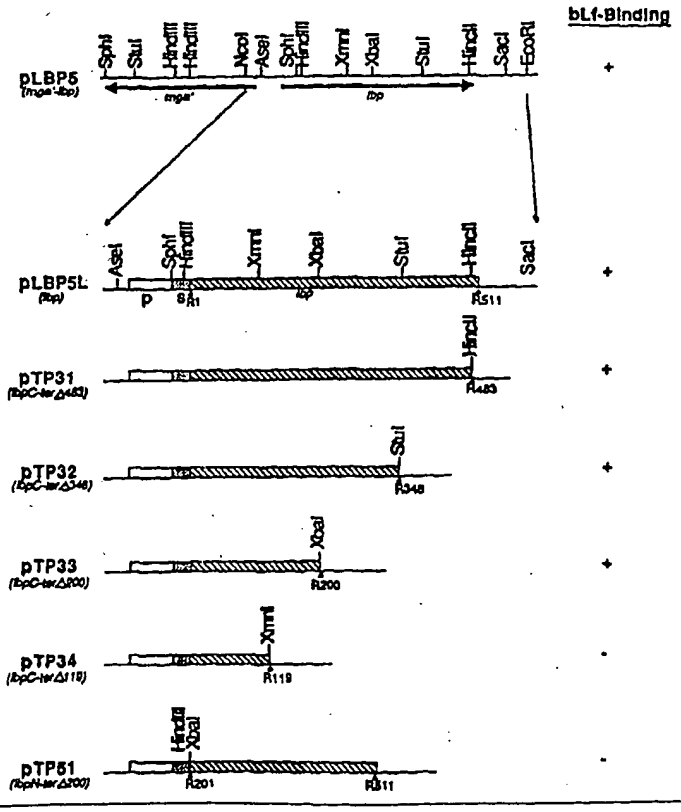
(54) Title: *STREPTOCOCCUS UBERIS* LACTOFERRIN–BINDING PROTEIN

(57) Abstract

The bovine lactoferrin (bLF) binding protein of *Streptococcus uberis (S. uberis)* is described, as well as the gene encoding the bLF (*lbp*). LF–binding proteins can be used in vaccine compositions for the prevention and treatment of *S. uberis* infections, particularly mastitis, as well as in diagnostic methods for determining the presence of *S. uberis* infections. Also disclosed is a regulatory region adjacent to *lbp*, termed *mga*.



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STREPTOCOCCUS UBERIS LACTOFERRIN-BINDING PROTEIN

Technical Field

The present invention relates generally to bacterial antigens. More particularly, the present invention pertains to the characterization and recombinant production of a bovine lactoferrin-binding protein from *Streptococcus uberis* (*S. uberis*) and the use of the same. The invention also relates to the characterization of a regulatory region, *mga*, located upstream of the lactoferrin-binding protein gene.

Background

Mastitis, an infection of the mammary gland, causes major economic losses to the dairy industry yearly. *Streptococcus uberis* (*S. uberis*) is an environmental pathogen responsible for a high proportion of cases of mastitis in lactating cows and is the predominant organism isolated from mammary glands during the nonlactating period (Bramley, A.J. (1984) *Br. Vet. J.* 140:328-335; Bramley and Dodd (1984) *J. Dairy Res.* 51:481-512; Oliver, S.P. (1988) *Am. J. Vet. Res.* 49:1789-1793). Mastitis resulting from infection with *S. uberis* is commonly subclinical, characterized by apparently normal milk with an increase in somatic cell counts due to the influx of leukocytes. The chemical composition of milk is changed due to suppression of secretion with the transfer of sodium chloride and bicarbonate from blood to milk, causing a shift of pH to a more alkaline level. *S. uberis* mastitis may also take the form of

an acute clinical condition, with obvious signs of disease such as clots or discoloration of the milk and swelling or hardness of the mammary gland. Some cases of the clinical disease can be severe and pyrexia may be present. For a review of the clinical manifestations of *S. uberis* mastitis, see, Bramley (1991) Mastitis: physiology or pathology. p. 3-9. In C. Burvenich, G. Vandeputte-van Messom, and A. W. Hill (ed.), *New insights into the pathogenesis of mastitis*. Rijksuniversiteit Gent, Belgium; and Schalm et al. (1971) The mastitis complex-A brief summary. p. 1-3. In *Bovine Mastitis*. Lea & Febiger, Philadelphia.

The pathogenesis of *S. uberis* infection is poorly understood. Furthermore, the influence of *S. uberis* virulence factors on host defense mechanisms and mammary gland physiology is not well defined. Known virulence factors associated with *S. uberis* include a hyaluronic acid capsule, hyaluronidase, R-like protein, plasminogen activator and CAMP factor. However, very little is known of their roles in pathogenicity.

Lactoferrin (Lf) is a mammalian iron-binding glycoprotein secreted by polymorphonuclear leukocytes (PMNs) and various exocrine glands (Baggiolini et al. (1970) *J. Exp. Med.* 131:559-570; Masson et al. (1966) *Clin. Chim. Acta* 14:735-739). This protein is found at high concentrations in milk and at mucosal surfaces (Masson et al., *supra*; Reiter and Oram (1967) *Nature* 216:328-330). For example, bovine lactoferrin (bLf) concentrations in lacteal secretions can increase up to 30-fold during acute bovine mastitis, depending on the severity of infection (Harmon et al. (1976) *Infect. Immun.* 13:533-542).

A regulatory function for Lf in various physiological pathways, including the adhesion of PMNs to the endothelial surface, feedback inhibition of the

granulocyte-monocyte colony-stimulating factor, and the regulation of antibody production, has been suggested. Specific interaction of Lf with certain mammalian cells seems to be involved in the above pathways and specific receptors for Lf have been identified on macrophages, monocytes, B lymphocytes, PMNs, activated T lymphocytes, and hepatocytes (Bennet and Davis (1981) *J. Immunol.* 127:1211-1216; Dehanne et al. (1985) *Am. J. Physiol.* 248:463-469; Maneva et al. (1983) *Int. J. Biochem.* 15:981-984; Rochard et al. (1989) *FEBS Lett.* 255:201-204; and van Snick and Masson (1976) *J. Exp. Med.* 144:1568-1580).

Lf inhibits the growth of *E. coli* and certain other microorganisms *in vitro* (Bullen et al. (1972) *Br. Med. J.* 1:69-75). This Lf-mediated antimicrobial action has mainly been attributed to its iron deprivation capacity with bacteria (Arnold et al. (1977) *Science* 197:263-265; Law and Reiter (1977) *J. Dairy Res.* 44:595-599; Oram and Reiter (1968) *Biochim. Biophys. Acta* 170:351-365). In this regard, it is well known that with few exceptions, iron is essential for microbial growth (Weinberg, E.D. (1978) *Microbiol. Rev.* 42:45-66. Even though iron is abundant within mammalian tissues, virtually all iron within the mammalian body is held intracellularly as ferritin or as heme compounds, pools which are generally inaccessible to invading microorganisms. Additionally, the small amount of iron present in extracellular spaces is effectively chelated by high-affinity iron-binding host glycoproteins such as transferrin (Tf), present in serum and lymph, and Lf, present in secretory fluids and milk (Otto et al. (1992) *Crit. Rev. Microbiol.* 18:217-233).

To overcome this deficiency, bacterial pathogens have developed specific iron uptake mechanisms. In many bacterial species, these

mechanisms involve the synthesis and secretion of small compounds called siderophores which display high affinity for ferric iron (FeIII). Siderophores are capable of removing TF- or Lf-bound iron to form ferrisiderophore complexes which in turn are recognized by specific iron-repressible membrane receptors and internalized into the bacterium where the iron is released (Crosa, J.H. (1989) *Microbiol. Rev.* 53:517-530). This iron uptake mechanism has been described for many gram-negative bacterial species. Some gram-negative bacteria do not secrete detectable siderophores when grown in an iron-deficient environment but produce outer membrane proteins that bind directly and specifically to Tf or Lf, thereby allowing iron transport into the bacterial cell.

Tf binding appears to be mediated by the activity of two proteins present in bacterial outer membranes, transferrin-binding protein 1 and 2 (Tbp1 and Tbp2) (Gonzalez et al. (1990) *Mol. Microbiol.* 4:1173-1179; Ogunnariwo and Schryvers (1990) *Infect. Immun.* 58:2091-2097; Schryvers, A.B. (1989) *J. Med. Microbiol.* 29:121-130); Schryvers and Lee (1989) *Can. J. Microbiol.* 35:409-415; Schryvers and Morris (1988) *Mol. Microbiol.* 2:467-472). Transferrin binding proteins tend to be highly specific for the transferrin of their natural host.

However, the mechanism of iron uptake from Lf has not been well characterized. A putative 105 kDa receptor for Lf utilization, Lbp1, has been identified in gonococcus by affinity isolation (Schryvers and Lee, *supra*; Cornelissen et al. (1992) *J. Bacteriol.* 174:5788-5797; Lee and Bryan (1989) *J. Med. Microbiol.* 28:199-204). The structural gene for Lbp1, termed *lbpA*, has been isolated (Biswas and Sparling (1995) *Infect. Immun.* 63:2958-2967). The genes for meningococcal lactoferrin receptors have

also been characterized (Petterson et al. (1993) *Infect. Immun.* 61:4724-4733; Petterson et al. (1994) *J. Bacteriol.* 176:1764-1766; Petterson et al. (1994) *Microb. Pathog.* 17:395-408). The DNA sequence of *lbpA* and the predicted amino acid sequence of Lbp1 in gonococcus and the meningococcus are highly conserved (94% identity). Lbp1 has been shown to be 46% identical to Tbp1 (Cornelissen et al. (1992) *J. Bacteriol.* 174:5788-5797) of the same gonococcal strain but only 18% identical to Tbp2 (Anderson et al. (1994) *J. Bacteriol.* 176:3162-3170). Both gonococcal and meningococcal genes contain relatively well-conserved Fur boxes and the proteins are homologous to the TonB-dependent family of receptors, as is true for Tbp1 (Cornelissen et al., *supra*) but not for Tbp2 (Anderson et al., *supra*). The strong similarity between the Lf receptor protein, Lbp1, and the Tf receptor protein, Tbp1, suggests that binding of Lf to bacterial cells might be similar to Tf binding. Consistent with this hypothesis is the fact that the putative protein encoded by *lbpB*, the open reading frame upstream of *lbpA*, shows extensive homology to Tbp2 (Pettersson et al., (1994) *Microb. Pathog.* 17:395-408), suggesting that iron-acquisition from Lf, as from Tf, requires two specific proteins in the outer membrane.

In contrast to the knowledge of the iron uptake systems of Gram-negative bacteria, there is comparatively little information concerning the mechanisms by which Gram-positive pathogens acquire iron during growth in extracellular body fluids. Both *S. aureus* and the coagulase-negative staphylococci have been reported to produce siderophores (Konetschny-Rapp et al. (1991) *Eur. J. Biochem.* 191:65-74; Meiwes et al. (1990) *FEMS Microbiol. Lett.* 67:201-206). *S. aureus* appears capable of binding

both human and bovine Lfs and human Tf (Naidu et al. (1991) *J. Med. Micrbiol.* 34:323-328; Naidu et al. (1991) *J. Dairy Sci.* 74:1218-1226; Naidu et al. (1992) *J. Med. Microbiol.* 36:177-183; Modun et al. (1994) *Infect. Immun.* 62:3850-3858). The interaction of Lf with a bovine *S. agalactiae* strain has also been reported (Rainard, P. (1992) *FEMS Microbiol. Lett.* 98:235-240). However, the iron acquisition functions of these Tf- or Lf-binding proteins have not been studied.

The group A streptococcal M protein is considered to be one of the major virulence factors of this organism by virtue of its ability to impede attack by human phagocytes (Lancefield, R.C. (1962) *J. Immunol.* 89:307-313). The bacteria persist in the infected tissue until antibodies are produced against the M molecule. Type-specific antibodies to the M protein are able to reverse the antiphagocytic effect of the molecule and allow efficient clearance of the invading organism. For example, M proteins are one of the key virulence factors of *S. pyogenes*, due to their involvement in mediating resistance to phagocytosis (Kehoe, M.A. (1991) *Vaccine* 9:797-806) and their ability to induce potentially harmful host immune responses via their superantigenicity and their capacity to induce host-cross-reactive antibody responses (Bisno, A.L. (1991) *New Engl. J. Med.* 325:783-793; Froude et al. (1989) *Curr. Top. Microbiol. Immunol.* 145:5-26; Stollerman, G.H. (1991) *Clin. Immunol. Immunopathol.* 61:131-142).

In group A streptococci (GAS), the genes for M protein (*emm*) as well as a peptidase (*scpA*) and, if present, genes encoding M protein-related IgG- and IgA-binding proteins (*fcrA* and *enn*, respectively) are clustered on the chromosome (Haanes et al. (1992) *J. Bacteriol.* 174:4967-4976; Hollingshead et al. (1993)

Mol. Microbiol. 8:707-717; Podbielski, A. (1993) *Mol. Gen. Genet.* 237:287-300). Expression of these virulence-associated surface proteins is co-regulated at the level of transcription by the protein Mga (which stands for multigene regulator of group A Streptococcus), formerly called Mry or VirR (Caparon and Scott (1987) *Proc. Natl. Acad. Sci. USA* 84:8677-8681; Chen et al. (1993) *Mol. Gen. Genet.* 241:685-693; Haanes and Cleary (1989) *J. Bacteriol.* 171:6397-6408; McIver et al. (1995) *J. Bacteriol.* 177:6619-6624; Perez-Casal et al. (1991) *J. Bacteriol.* 173:2617-2624; Podbielski et al. (1995) *Infect. Immun.* 63:9-20; Podbielski, A. (1992) *Med. Microbiol. Immunol.* 181:227-240; Robbins et al. (1987) *J. Bacteriol.* 169:5633-5640). It is thought that Mga is a part of a crucial regulatory system in GAS, possibly functioning as a second component in a two-component regulatory system.

Vaccination is one approach to enhance resistance of the mammary gland to new infection and reduce clinical severity of the disease. Previous studies have shown that primary infection with *S. uberis* can considerably reduce the rate of infection following a second challenge with the same strain (Hill, A.W. (1988) *Res.Vet. Sci.* 44:386-387). Local vaccination with killed *S. uberis* protects the bovine mammary gland against intramammary challenge with the homologous strain (Finch et al. (1994) *Infect. Immun.* 62:3599-3603). Similarly, subcutaneous vaccination with live *S. uberis* has been shown to cause a dramatic modification of the pathogenesis of mastitis with the same strain (Hill et al. (1994) *FEMS Immunol. Med. Microbiol.* 8:109-118). Animals vaccinated in this way shed fewer bacteria in their milk and many quarters remain free of infection.

However, vaccination with live or attenuated bacteria can pose risks to the recipient. It would therefore be desirable to provide a subunit vaccine composition for use against *S. uberis*. Until now, the *S. uberis* lactoferrin-binding protein has not been characterized and it use in vaccine compositions has not been described.

Disclosure of the Invention

The present invention is based on the discovery of a bovine lactoferrin (bLF) binding protein (bLbp) from *S. uberis*, and the characterization thereof. The gene coding for bLF-binding protein, *lbp*, as well as an upstream regulator of the gene, *mga*, have been cloned. bLF-binding protein, immunogenic fragments and analogs thereof, and/or chimeric proteins including the same, can be used, either alone or in combination with other antigens, in novel subunit vaccines to provide protection from bacterial infection in mammalian subjects.

Accordingly, in one embodiment, the subject invention is directed to an isolated, immunogenic *S. uberis* bLF-binding protein, as well as a nucleic acid molecule comprising a coding sequence for an immunogenic *S. uberis* bLF-binding protein. In additional embodiments, the invention is directed to recombinant vectors including the same, host cells transformed with these vectors and methods of recombinantly producing *S. uberis* bLF-binding proteins.

In still further embodiments, the subject invention is directed to vaccine compositions comprising a pharmaceutically acceptable vehicle and an immunogenic *S. uberis* bLF-binding protein.

In yet other embodiments, the present invention is directed to methods of treating or preventing *S. uberis* infections, such as mastitis, in a mammalian subject. The method comprises administering to the subject a therapeutically effective amount of the above vaccine compositions.

In additional embodiments, the invention pertains to methods of producing vaccine compositions comprising (a) providing an immunogenic *S. uberis* bLF-binding protein; and (b) combining the protein with a pharmaceutically acceptable vehicle.

In further embodiments, the invention is directed to antibodies against the S. uberis bLF-binding proteins.

In additional embodiments, the invention is directed to methods of detecting *S. uberis* antibodies in a biological sample comprising:

(a) providing a biological sample;

(b) reacting the biological sample with a *S. uberis* bLF-binding protein under conditions which allow *S. uberis* antibodies, when present in the biological sample, to bind to the *S. uberis* bLF-binding protein to form an antibody/antigen complex; and

(c) detecting the presence or absence of the complex,

thereby detecting the presence or absence of *S. uberis* antibodies in the sample.

In yet further embodiments, the invention is directed to an immunodiagnostic test kit for detecting *S. uberis* infection. The test kit comprises a *S. uberis* bLF-binding protein and instructions for conducting the immunodiagnostic test.

In further embodiments, the invention is directed to an isolated *S. uberis* Mga protein, as well

as a nucleic acid molecule comprising a coding sequence for the same.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figure 1 is a restriction enzyme map of *lbp* and shows progressive deletions and a summary of bLf binding data. Open boxes (p region) represent 5'-sequences containing promoter and ribosome binding sites, shaded boxes (s region) represent lbp sequences encoding the signal peptides, and hatched boxes represent the *lbp* sequences coding for the mature or truncated proteins. R1 and R201 (R represents residue) indicate the first codon of the mature protein in pLBP5 and the truncated protein in pTP51, respectively. Other numbers indicate the last codon of each protein. Restriction sites are present on the top of the *lbp*. The bLf binding ability of each clone is shown on the right.

Figures 2A-2C (SEQ ID NOS:1-2) depict the nucleotide sequence and deduced amino acid sequence of *S. uberis* bovine Lbp. Nucleotides and amino acids are numbered on the right of the sequences. The deduced amino acid sequence is shown in the single-letter code below the nucleotide sequence. Two possible ATG start codons at positions 232 and 262, and the TAA stop codon at 1915, are shown in bold. Two putative -35 and -10 promoter sequences and Shine-Dalgarno sequences (SD) are indicated. A putative rho-independent transcription terminator (T) is underlined. The double underline shows the presence of a putative signal peptide at the N-terminus of the ORF. The C-terminal hydrophobic trans-membrane domain is indicated by italics and the nearby surface anchor

motif is shown by italics and double underline. The central repeated amino acid sequences are indicated by the letters A, B, and C.

Figure 3 shows profiles of the secondary structures, charged residues and hydrophobicity of Lbp. The deduced amino acid sequence of Lbp was analyzed with the Novotny-Auffray algorithm. Plots marked Turn, Beta, and Alpha indicate the potential for beta turn-random coil, beta sheet, and alpha helix formation, respectively. The +/- plot shows regions of the molecule with net positive (upper) and negative (lower) charges. The hydrophobicity (Hydro) plot shows the hydrophobic regions of the protein. The positions of the amino acids are shown on the horizontal axis.

Figure 4 depicts the construction of pMGA14F from pLBP5i and pMGA14. Plasmid pMGA14F was generated by inserting the 1.5 kb *Sph*I-*Nhe*I fragment of pLBP5i into the *Sph*I and *Nhe*I sites of pMGA14. Lines indicate *S. uberis* DNA, while the box represents the multiple cloning sites of vector pTZ18R. The probe fragments used for Southern or Northern blot experiments are indicated by the hatched bars. The arrows indicate the locations of the open reading frames of *lbp*, *mga'* and *mga*.

Figures 5A-5D (SEQ ID NOS:3-12) show the nucleotide sequence of *mga* and deduced amino acid sequence of Mga, as well as the ORFs downstream of *mga*. Nucleotides and amino acids are numbered on the right of the sequences. The deduced amino acid sequence is shown in the single-letter code below the nucleotide sequence. Possible ATG start codons are shown in bold and the stop codons are indicated by "*". A putative -35 and -10 promoter sequence and Shine-Dalgarno sequence (SD) are indicated.

Figure 6 shows the probes used in hybridization analysis, as well as the structure of Lbp (on the top), constructed from the DNA sequence analysis. The signal peptide, proline rich region and transmembrane domain are indicated by S, Pro and TM respectively. The A, B and C repeat regions are also shown. Probes used in hybridization are indicated by the hatched bars below the map.

Figure 7 shows the time course of ^{125}I-bLf binding to *S. uberis*. 10^9 bacteria were incubated with 6.9 nm ^{125}I-bLf in 0.2 ml of PBS-1% BSA. At time intervals indicated, bacteria were pelleted and the amount of cell associated ^{125}I-bLf was determined.

Figure 8 depicts the results of a competition binding assay using bLf (33% iron-saturated) as radiolabelled ligand and competitor. Percentage binding values were calculated as percentage of ^{125}I-bLf binding in the presence of increasing amounts of unlabelled bLf to bacteria suspended in PBS-1% BSA in the absence of unlabelled bLf. Inset: Scatchard plot and affinity (Kd) of the binding of ^{125}I-bLf to *S. uberis*. The line represents the best fit as determined by a linear regression analysis. A concentration of 270 nM of unlabelled bLf caused 50% displacement of ^{125}I-bLf binding (indicated by dotted lines).

Figure 9 shows the influence of iron chelators on the expression of lactoferrin-binding by *S. uberis*. Cells grown in THB-YE with or without EDDA, dipyridyl or desferrioxamine mesylate were incubated with 6.9 nM ^{125}I-bLf in 0.2 ml of PBS-1% BSA at room temperature for 2 h. After three washes, cell-bound radioactivity was determined.

Figure 10 shows the physical map of the recombinant plasmid used to express the Lbp in Example 3. The plasmid pLBP5 contains 3.7 kb of *S. uberis*-

derived kDNA, in the vector pTZ18R. The plasmid pGH-LBP was constructed by subcloning an *SphI-RsaI* fragment from pLBP5 into vector pGH433. P_{tac} indicates the location of the *tac* promoter. The Lbp gene is shown by the arrows labelled as *lbp*.

Figure 11 shows inhibition of recombinant bLf-binding protein on ^{125}I-labelled bLf binding to *S. uberis*. Increasing amounts of a mixture of supernatant (sup.) and whole cell lysate (with equal volume) of *E. coli* pLBP5 (●) or *E. coli* pTZ18R (○) were mixed with 10^9 cells and incubated with 0.69 nM ^{125}I-bLf in 0.2 ml volumes. Inhibition values were calculated as relative percentage of bLf binding to bacteria suspended in PBS-1% BSA in the absence of any *E. coli* samples.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Vols. I, II and III, Second Edition (1989); *DNA Cloning*, Vols. I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds. 1984); *Animal Cell Culture* (R.K. Freshney ed. 1986); *Immobilized Cells and Enzymes* (IRL press, 1986); Perbal, B., *A Practical Guide to Molecular Cloning* (1984); the series, *Methods In Enzymology* (S. Colowick and N. Kaplan eds., Academic Press, Inc.); and *Handbook of Experimental Immunology*, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications).

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

It must be noted that, as used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an Lbp" includes a mixture of two or more Lbps, and the like.

The terms "lactoferrin-binding protein", "LF-binding protein" and "Lbp" (used interchangeably herein) or a nucleotide sequence encoding the same, intends a protein or a nucleotide sequence, respectively, which is derived from an *S. uberis lbp* gene. The nucleotide sequence of a representative *S. uberis lbp* gene, and the corresponding amino acid sequence of an LF-binding protein encoded by this gene, are depicted in Figures 2A-2C (SEQ ID NOS:1-2). However, an LF-binding protein as defined herein is not limited to the depicted sequence as several subtypes of *S. uberis* are known and variations in LF-binding proteins will occur between strains of *S. uberis*.

Furthermore, the derived protein or nucleotide sequences need not be physically derived from the gene described above, but may be generated in any manner, including for example, chemical synthesis, isolation (e.g., from *S. uberis*) or by recombinant production, based on the information provided herein. Additionally, the term intends proteins having amino acid sequences substantially homologous (as defined below) to contiguous amino acid sequences encoded by the genes, which display immunological and/or lactoferrin-binding activity.

Thus, the terms intend full-length, as well as immunogenic, truncated and partial sequences, and active analogs and precursor forms of the proteins. Also included in the term are nucleotide fragments of the gene that include at least about 8 contiguous base pairs, more preferably at least about 10-20 contiguous base pairs, and most preferably at least about 25 to 50 or more contiguous base pairs of the gene. Such fragments are useful as probes and in diagnostic methods, discussed more fully below.

The terms also include those forms possessing, as well as lacking, the signal sequence, as well as the nucleic acid sequences coding therefor. Additionally, the term intends forms of LF-binding protein which lack the membrane anchor region, and nucleic acid sequences encoding such deletions. Such deletions may be desirable in systems that do not provide for secretion of the protein. Furthermore, an LF-binding domain, found within about the N-terminal 200 codons, may or may not be present. Thus, for example, if the Lf binding protein will be used to purify LF, the LF-binding domain will generally be retained. If the protein is to be used in vaccine compositions, immunogenic epitopes which may or may not include the LF-binding domain, will be present.

The terms also include proteins in neutral form or in the form of basic or acid addition salts depending on the mode of preparation. Such acid addition salts may involve free amino groups and basic salts may be formed with free carboxyls. Pharmaceutically acceptable basic and acid addition salts are discussed further below. In addition, the proteins may be modified by combination with other biological materials such as lipids (both those occurring naturally with the molecule or other lipids that do not destroy immunological activity) and

saccharides, or by side chain modification, such as acetylation of amino groups, phosphorylation of hydroxyl side chains, oxidation of sulfhydryl groups, glycosylation of amino acid residues, as well as other modifications of the encoded primary sequence.

The term therefore intends deletions, additions and substitutions to the sequence, so long as the polypeptide functions to produce an immunological response as defined herein. In this regard, particularly preferred substitutions will generally be conservative in nature, i.e., those substitutions that take place within a family of amino acids. For example, amino acids are generally divided into four families: (1) acidic -- aspartate and glutamate; (2) basic -- lysine, arginine, histidine; (3) non-polar -- alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar -- glycine, asparagine, glutamine, cystine, serine threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified as aromatic amino acids. For example, it is reasonably predictable that an isolated replacement of leucine with isoleucine or valine, or vice versa; an aspartate with a glutamate or vice versa; a threonine with a serine or vice versa; or a similar conservative replacement of an amino acid with a structurally related amino acid, will not have a major effect on the biological activity. Proteins having substantially the same amino acid sequence as the reference molecule, but possessing minor amino acid substitutions that do not substantially affect the immunogenicity of the protein, are therefore within the definition of the reference polypeptide.

By "mastitis" is meant an inflammation of the mammary gland in mammals, including in cows, ewes, goats, sows, mares, and the like, caused by the

presence of *S. uberis*. The infection manifests itself by the infiltration of phagocytic cells in the gland. Generally, 4 clinical types of mastitis are recognized: (1) peracute, associated with swelling, heat, pain, and abnormal secretion in the gland and accompanied by fever and other signs of systemic disturbance, such as marked depression, rapid weak pulse, sunken eyes, weakness and complete anorexia; (2) acute, with changes in the gland similar to those above but where fever, anorexia and depression are slight to moderate; (3) subacute, where no systemic changes are displayed and the changes in the gland and its secretion are less marked: and (4) subclinical, where the inflammatory reaction is detectable only by standard tests for mastitis.

Standard tests for the detection of mastitis include but are not limited to, the California Mastitis Test, the Wisconsin Mastitis Test, the Nagase test, the electronic cell count and somatic cell counts used to detect a persistently high white blood cell content in milk. In general, a somatic cell count of about 300,000 to about 500,000 cells per ml or higher, in milk will indicate the presence of infection. Thus, a vaccine is considered effective in the treatment and/or prevention of mastitis when, for example, the somatic cell count in milk is retained below about 500,000 cells per ml. For a discussion of mastitis and the diagnosis thereof, see, e.g., *The Merck Veterinary Manual. A Handbook of Diagnosis, Therapy, and Disease Prevention and Control for the Veterinarian*, Merck and Co., Rahway, New Jersey, 1991.

An "isolated" nucleic acid molecule is a nucleic acid molecule separate and discrete from the whole organism with which the molecule is found in nature; or a nucleic acid molecule devoid, in whole or part, of sequences normally associated with it in

nature; or a sequence, as it exists in nature, but having heterologous sequences (as defined below) in association therewith.

By "subunit vaccine composition" is meant a composition containing at least one immunogenic polypeptide, but not all antigens, derived from or homologous to an antigen from a pathogen of interest. Such a composition is substantially free of intact pathogen cells or particles, or the lysate of such cells or particles. Thus, a "subunit antigen composition" is prepared from at least partially purified (preferably substantially purified) immunogenic polypeptides from the pathogen, or recombinant analogs thereof. A subunit antigen composition can comprise the subunit antigen or antigens of interest substantially free of other antigens or polypeptides from the pathogen.

The term "epitope" refers to the site on an antigen or hapten to which specific B cells and/or T cells respond. The term is also used interchangeably with "antigenic determinant" or "antigenic determinant site." Antibodies that recognize the same epitope can be identified in a simple immunoassay showing the ability of one antibody to block the binding of another antibody to a target antigen.

An "immunological response" to a composition or vaccine is the development in the host of a cellular and/ or antibody-mediated immune response to the composition or vaccine of interest. Usually, an "immunological response" includes but is not limited to one or more of the following effects: the production of antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells and/or $\gamma\delta$ T cells, directed specifically to an antigen or antigens included in the composition or vaccine of interest. Preferably, the host will display either a

therapeutic or protective immunological response such that resistance of the mammary gland to new infection will be enhanced and/or the clinical severity of the disease reduced. Such protection will be demonstrated by either a reduction or lack of symptoms normally displayed by an infected host, a quicker recovery time and/or a lowered somatic cell count in milk from the infected quarter.

The terms "immunogenic" protein or polypeptide refer to an amino acid sequence which elicits an immunological response as described above. An "immunogenic" protein or polypeptide, as used herein, includes the full-length sequence of the LF-binding protein, with or without the signal sequence, membrane anchor domain and/or LF-binding domain, analogs thereof, or immunogenic fragments thereof. By "immunogenic fragment" is meant a fragment of an LF-binding protein which includes one or more epitopes and thus elicits the immunological response described above. Such fragments can be identified using any number of epitope mapping techniques, well known in the art. See, e.g., *Epitope Mapping Protocols* in Methods in Molecular Biology, Vol. 66 (Glenn E. Morris, Ed., 1996) Humana Press, Totowa, New Jersey. For example, linear epitopes may be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Patent No. 4,708,871; Geysen et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:3998-4002; Geysen et al. (1986) *Molec. Immunol.* 23:709-715. Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray

crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., *Epitope Mapping Protocols, supra*.

Immunogenic fragments, for purposes of the present invention, will usually include at least about 3 amino acids, preferably at least about 5 amino acids, more preferably at least about 10-15 amino acids, and most preferably 25 or more amino acids, of the Lbp molecule. There is no critical upper limit to the length of the fragment, which could comprise nearly the full-length of the protein sequence, or even a fusion protein comprising two or more epitopes of Lbp.

"Native" proteins or polypeptides refer to proteins or polypeptides isolated from the source in which the proteins naturally occur. "Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic" polypeptides are those prepared by chemical synthesis.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A DNA "coding sequence" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory elements. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g.,

mammalian) DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding sequence.

DNA "control elements" refers collectively to promoters, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell. Not all of these control sequences need always be present in a recombinant vector so long as the desired gene is capable of being transcribed and translated.

"Operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control elements operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control elements need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter and the coding sequence and the promoter can still be considered "operably linked" to the coding sequence.

A control element, such as a promoter, "directs the transcription" of a coding sequence in a cell when RNA polymerase will bind the promoter and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an exogenous nucleic acid molecule.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be

integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In procaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the exogenous DNA.

"Homology" refers to the percent identity between two polynucleotide or two polypeptide moieties. The correspondence between the sequence from one moiety to another can be determined by techniques known in the art. For example, homology can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs such as ALIGN, Dayhoff, M.O. (1978) in *Atlas of Protein Sequence and Structure* 5:Suppl. 3, National biomedical Research Foundation, Washington, DC.

Alternatively, homology can be determined by hybridization of polynucleotides under conditions which form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when the sequences exhibit at least about 80%-85%, preferably at least about 90%, and most preferably at least about 95%-98% sequence identity over a defined length of the molecules, as determined using the methods above. As used herein, substantially homologous also refers to

sequences showing complete identity to the specified DNA or polypeptide sequence. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Sambrook et al., *supra*; *DNA Cloning*, *supra*; *Nucleic Acid Hybridization*, *supra*.

The term "functionally equivalent" intends that the amino acid sequence of an LF-binding protein is one that will elicit a substantially equivalent or enhanced immunological response, as defined above, as compared to the response elicited by an LF-binding protein having identity with the reference LF-binding protein, or an immunogenic portion thereof.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source bacteria. Another example of the heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

The term "treatment" as used herein refers to either (i) the prevention of infection or reinfection (prophylaxis), or (ii) the reduction or elimination of symptoms of the disease of interest (therapy).

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from a subject, including but not limited to, for example, blood, plasma, serum, fecal matter, urine, bone marrow, bile, spinal fluid, lymph fluid, samples of the skin, external secretions of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, organs, biopsies and also samples of *in vitro* cell culture constituents including but not limited to conditioned media resulting from the growth of cells and tissues in culture medium, e.g., recombinant cells, and cell components.

As used herein, the terms "label" and "detectable label" refer to a molecule capable of detection, including, but not limited to, radioactive isotopes, fluorescers, chemiluminescers, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, chromophores, dyes, metal ions, metal sols, ligands (e.g., biotin or haptens) and the like. The term "fluorescer" refers to a substance or a portion thereof which is capable of exhibiting fluorescence in the detectable range. Particular examples of labels which may be used under the invention include fluorescein, rhodamine, dansyl, umbelliferone, Texas red, luminol, NADPH and α-β-galactosidase.

B. General Methods

Central to the present invention is the discovery of a bovine LF-binding protein in *S. uberis*. The gene for the *S. uberis* bLF-binding protein ("*lbp*") has been isolated and characterized, and the protein encoded thereby sequenced. The complete DNA and amino acid sequences of *S. uberis* bLF-binding protein are shown in Figures 2A-2C (SEQ ID NOS:1-2). In

particular, as described in the examples, a single ORF of 1683 bp, depicted as residues 232-1914, inclusive of Figures 2A-2C (SEQ ID NOS:1-2), encoding 561 amino acid residues, gives rise to two protein species able to bind bovine lactoferrin, having molecular weights of 76 kDa and 165 kDa, respectively. The 165 kDa protein is likely a dimer of the 76 kDa protein since urea treatment results in a single band and Northern blot analysis shows only one major transcript in *S. uberis*, as well as in recombinant *E. coli* transformed with a construct encoding the LF-binding protein.

S. uberis bovine LF-binding protein includes a putative N-terminal signal peptide of about 50 amino acids (if translation starts at the first ATG codon shown in Figure 2A). Thus, the full-length bovine LF-binding protein depicted, including the signal sequence, is found at amino acid positions 1-561, inclusive, (encoded by nucleotide positions 232-1914, inclusive) of Figures 2A-2C (SEQ ID NOS:1-2). The mature protein, lacking the signal peptide, is found at amino acid positions 52-561, inclusive, (nucleotide positions 445-1914, inclusive) of Figures 2A-2C. A membrane anchor motif at the C-terminus is also present, as indicated in Figures 2A-2C (SEQ ID NOS:1-2). A bovine Lf binding domain is present in a 200 codon N-terminal region of the molecule. The protein appears to lack disulfide bridges.

As shown in the examples, the binding of ^{125}I-bLf to *S. uberis* was time-dependent and displaceable by unlabelled bLf. Apo-bLf inhibits ^{125}I-bLf binding as effectively as iron-saturated bLf. Bovine transferrin, human lactoferrin and human transferrin do not interfere with bLf binding. The Scatchard plot is linear and approximately 7800 binding sites are expressed by each bacterial cell, with an affinity of 1.0×10^{-7} M. Reduced iron

availability does not significantly modify the saturation of *S. uberis* by bLf. The bLf binding protein described herein is lactoferrin species-specific, in that human Lf does not appear to effectively block the binding of bovine Lf.

The Lf binding protein of *S. uberis* differs from the transferrin receptors of *Haemophilus* and *Neisseria* spp., which consist of two distinct transferrin-binding proteins, termed Tbp1 and Tbp2, which range in molecular weight from 68 to 105 kDa depending on the strain. Similarly, the bovine Lf receptor of *S. aureus* consists of two distinct bLf binding proteins with estimated molecular weights of 92 and 67 kDa (Naidu et al. (1991) *J. Dairy Sci.* 74:1218-1226) and therefore appears to be different from the receptor described herein. Also, the streptococcal LF-binding protein described herein appears to be different from the *S. aureus* human Lf binding protein, an approximately 450 kDa protein which, under reducing SDS-PAGE gel conditions, resolves into two components of 67 and 62 kDa.

Analysis of the primary and secondary structure of the *S. uberis* bLf binding protein suggests that it is an M-like protein. In particular, a gene homologous to the group A streptococcal *mga*, a positive regulator of M and M-like proteins, has been found in the upstream adjacent region of *lbp*. Southern blot analysis reveals that *mga* is present in all *S. uberis* strains tested that contained the *lbp*.

The sequence of *S. uberis mga* and the protein product therefrom is presented in Figures 5A-5D (SEQ ID NOS:3-12). Starting at the ATG initiation codon at nucleotides 361-363 and terminating at a TAA codon at nucleotides 1858-1860, the deduced gene product, Mga, is comprised of 499 amino acid residues with a calculated molecular weight of 58,454 Da. The

N-terminus of Mga lacks the features of a signal peptide, suggesting that it is a cytoplasmic protein. Preceding the start codon of *mga* is a putative ribosome binding site AGGAGA. Sequences resembling the -35 and -10 promoter motifs have also been identified, as shown in Figures 5A-5D (SEQ ID NOS:3-12).

S. uberis LF-binding protein, immunogenic fragments thereof or chimeric proteins including the same, can be provided in subunit vaccine compositions to treat or prevent bacterial infections caused by *S. uberis*, including mastitis in mammals, such as in bovine, equine, ovine and goat species. In addition to use in vaccine compositions, the proteins and fragments thereof, antibodies thereto, and genes coding therefor, can be used as diagnostic reagents to detect the presence of infection in a mammalian subject. Similarly, the genes encoding the proteins can be cloned and used to design probes to detect and isolate homologous genes in other bacterial strains. For example, fragments comprising at least about 15-20 nucleotides, more preferably at least about 20-50 nucleotides, and most preferably about 60-100 or more nucleotides, will find use in these embodiments. The *S. uberis* LF-binding proteins also find use in purifying bovine LFs from streptococcal species and from recombinant host cells expressing the same.

S. uberis Lf binding proteins can be used in vaccine compositions either alone or in combination with other bacterial, fungal, viral or protozoal antigens. These antigens can be provided separately or even as fusion proteins comprising one or more epitopes of an LF-binding protein fused to one or more of these antigens. For example, other immunogenic proteins from *S. uberis*, such as the CAMP factor, hyaluronic acid capsule, hyaluronidase, R-like protein

and plasminogen activator, can be administered with the LF-binding protein. Additionally, immunogenic proteins from other organisms involved in mastitis, such as from the genera *Staphylococcus*, *Corynebacterium*, *Pseudomonas*, *Nocardia*, *Clostridium*, *Mycobacterium*, *Mycoplasma*, *Pasteurella*, *Prototheca*, other streptococci, coliform bacteria, as well as yeast, can be administered along with the bLF-binding proteins described herein to provide a broad spectrum of protection. Thus, for example, immunogenic proteins from *Staphylococcus aureus*, *Str. agalactiae*, *Str. dysgalactiae*, *Str. zooepidemicus*, *Corynebacterium pyogenes*, *Pseudomonas aeruginosa*, *Nocardia asteroides*, *Clostridium perfringens*, *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella spp.* can be provided along with the bLF-binding proteins of the present invention.

Production of LF-Binding Protein

The above described LF-binding proteins and active fragments, analogs and chimeric proteins derived from the same, can be produced by a variety of methods. Specifically, LF-binding proteins can be isolated directly from bacteria which express the same. This is generally accomplished by first preparing a crude extract which lacks cellular components and several extraneous proteins. The desired proteins can then be further purified i.e. by column chromatography, HPLC, immunoadsorbent techniques or other conventional methods well known in the art.

Alternatively, the proteins can be recombinantly produced as described herein. As explained above, these recombinant products can take the form of partial protein sequences, full-length sequences, precursor forms that include signal

sequences, mature forms without signals, or even fusion proteins (e.g., with an appropriate leader for the recombinant host, or with another subunit antigen sequence for *Streptococcus* or another pathogen).

The *lbp* genes of the present invention can be isolated based on the ability of the protein products to bind LF, using LF-binding assays as described below. Thus, gene libraries can be constructed and the resulting clones used to transform an appropriate host cell. Colonies can be pooled and screened for clones having LF-binding activity. Colonies can also be screened using polyclonal serum or monoclonal antibodies to the LF-binding protein.

Alternatively, once the amino acid sequences are determined, oligonucleotide probes which contain the codons for a portion of the determined amino acid sequences can be prepared and used to screen genomic or cDNA libraries for genes encoding the subject proteins. The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those of ordinary skill in the art. See, e.g., *DNA Cloning*: Vol. I, *supra*; *Nucleic Acid Hybridization*, *supra*; *Oligonucleotide Synthesis*, supra; Sambrook et al., *supra*. Once a clone from the screened library has been identified by positive hybridization, it can be confirmed by restriction enzyme analysis and DNA sequencing that the particular library insert contains an LF-binding protein gene or a homolog thereof. The genes can then be further isolated using standard techniques and, if desired, PCR approaches or restriction enzymes employed to delete portions of the full-length sequence.

Similarly, genes can be isolated directly from bacteria using known techniques, such as phenol extraction and the sequence further manipulated to

produce any desired alterations. See, e.g., Sambrook et al., *supra*, for a description of techniques used to obtain and isolate DNA.

Alternatively, DNA sequences encoding the proteins of interest can be prepared synthetically rather than cloned. The DNA sequences can be designed with the appropriate codons for the particular amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) *Nature* 292:756; Nambair et al. (1984) *Science* 223:1299; Jay et al. (1984) *J. Biol. Chem.* 259:6311.

Once coding sequences for the desired proteins have been prepared or isolated, they can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage λ (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pHV14 (*E. coli* and *Bacillus subtilis*), pBD9 (*Bacillus*), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5 (*Saccharomyces*), YCp19 (*Saccharomyces*) and bovine papilloma virus (mammalian cells). See, Sambrook et al., *supra*; *DNA Cloning*, *supra*; B. Perbal, *supra*.

The gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the

DNA sequence encoding the desired protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. If a signal sequence is included, it can either be the native, homologous sequence, or a heterologous sequence. For example, the signal sequence for *S. uberis* LF-binding protein (shown in Figure 2A), can be used for secretion thereof, as can a number of other signal sequences, well known in the art. Leader sequences can be removed by the host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

Other regulatory sequences may also be desirable which allow for regulation of expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases it may be necessary to modify the coding sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the proper reading frame. It may also be desirable to produce mutants or analogs of the

LF-binding protein. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis, are described in, e.g., Sambrook et al., *supra*; *DNA Cloning*, *supra*; *Nucleic Acid Hybridization*, *supra*.

The expression vector is then used to transform an appropriate host cell. A number of mammalian cell lines are known in the art and include immortalized cell lines available from the American Type Culture Collection (ATCC), such as, but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), Madin-Darby bovine kidney ("MDBK") cells, as well as others. Similarly, bacterial hosts such as *E. coli*, *Bacillus subtilis*, and *Streptococcus spp.*, will find use with the present expression constructs. Yeast hosts useful in the present invention include *inter alia*, *Saccharomyces cerevisiae*, *Candida albicans*, *Candida maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis*, *Kluyveromyces lactis*, *Pichia guillerimondii*, *Pichia pastoris*, *Schizosaccharomyces pombe* and *Yarrowia lipolytica*. Insect cells for use with baculovirus expression vectors include, *inter alia*, *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*.

Depending on the expression system and host selected, the proteins of the present invention are produced by culturing host cells transformed by an expression vector described above under conditions

whereby the protein of interest is expressed. The protein is then isolated from the host cells and purified. If the expression system secretes the protein into the growth media, the protein can be purified directly from the media. If the protein is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

The proteins of the present invention may also be produced by chemical synthesis such as solid phase peptide synthesis, using known amino acid sequences or amino acid sequences derived from the DNA sequence of the genes of interest. Such methods are known to those skilled in the art. See, e.g., J. M. Stewart and J. D. Young, *Solid Phase Peptide Synthesis*, 2nd Ed., Pierce Chemical Co., Rockford, IL (1984) and G. Barany and R. B. Merrifield, *The Peptides: Analysis, Synthesis, Biology*, editors E. Gross and J. Meienhofer, Vol. 2, Academic Press, New York, (1980), pp. 3-254, for solid phase peptide synthesis techniques; and M. Bodansky, *Principles of Peptide Synthesis*, Springer-Verlag, Berlin (1984) and E. Gross and J. Meienhofer, Eds., *The Peptides: Analysis, Synthesis, Biology, supra*, Vol. 1, for classical solution synthesis. Chemical synthesis of peptides may be preferable if a small fragment of the antigen in question is capable of raising an immunological response in the subject of interest.

The LF-binding proteins of the present invention, or their fragments, can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) is immunized with an antigen of the present invention, or its fragment, or a mutated antigen. Serum from the immunized animal is collected and treated according to

known procedures. See, e.g., Jurgens et al. (1985) *J. Chrom.* 348:363-370. If serum containing polyclonal antibodies is used, the polyclonal antibodies can be purified by immunoaffinity chromatography, using known procedures.

Monoclonal antibodies to the LF-binding proteins and to the fragments thereof, can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by using hybridoma technology is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al., *Hybridoma Techniques* (1980); Hammerling *et al.*, *Monoclonal Antibodies and T-cell Hybridomas* (1981); Kennett et al., *Monoclonal Antibodies* (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500, 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the LF-binding protein, or fragment thereof, can be screened for various properties; i.e., for isotype, epitope, affinity, etc. Monoclonal antibodies are useful in purification, using immunoaffinity techniques, of the individual antigens which they are directed against. Both polyclonal and monoclonal antibodies can also be used for passive immunization or can be combined with subunit vaccine preparations to enhance the immune response. Polyclonal and monoclonal antibodies are also useful for diagnostic purposes.

Vaccine Formulations and Administration

The LF-binding proteins of the present invention can be formulated into vaccine compositions,

either alone or in combination with other antigens, for use in immunizing subjects as described below. Methods of preparing such formulations are described in, e.g., *Remington's Pharmaceutical Sciences*, Mack Publishing Company, Easton, Pennsylvania, 18 Edition, 1990. Typically, the vaccines of the present invention are prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in or suspension in liquid vehicles prior to injection may also be prepared. The preparation may also be emulsified or the active ingredient encapsulated in liposome vehicles. The active immunogenic ingredient is generally mixed with a compatible pharmaceutical vehicle, such as, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vehicle may contain minor amounts of auxiliary substances such as wetting or emulsifying agents and pH buffering agents.

Adjuvants which enhance the effectiveness of the vaccine may also be added to the formulation. Adjuvants may include for example, muramyl dipeptides, avridine, aluminum hydroxide, dimethyldioctadecyl ammonium bromide (DDA), oils, oil-in-water emulsions, saponins, cytokines, and other substances known in the art.

The Lf binding protein may be linked to a carrier in order to increase the immunogenicity thereof. Suitable carriers include large, slowly metabolized macromolecules such as proteins, including serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those skilled in the art; polysaccharides, such as sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids such as polyglutamic acid, polylysine, and

the like; amino acid copolymers; and inactive virus particles.

The LF-binding proteins may be used in their native form or their functional group content may be modified by, for example, succinylation of lysine residues or reaction with Cys-thiolactone. A sulfhydryl group may also be incorporated into the carrier (or antigen) by, for example, reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(4-dithiopyridyl propionate. Suitable carriers may also be modified to incorporate spacer arms (such as hexamethylene diamine or other bifunctional molecules of similar size) for attachment of peptides.

Other suitable carriers for the LF-binding proteins of the present invention include VP6 polypeptides of rotaviruses, or functional fragments thereof, as disclosed in U.S. Patent No. 5,071,651. Also useful is a fusion product of a viral protein and the subject immunogens made by methods disclosed in U.S. Patent No. 4,722,840. Still other suitable carriers include cells, such as lymphocytes, since presentation in this form mimics the natural mode of presentation in the subject, which gives rise to the immunized state. Alternatively, the proteins of the present invention may be coupled to erythrocytes, preferably the subject's own erythrocytes. Methods of coupling peptides to proteins or cells are known to those of skill in the art.

Furthermore, the LF-binding proteins (or complexes thereof) may be formulated into vaccine compositions in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or

phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

Vaccine formulations will contain a "therapeutically effective amount" of the active ingredient, that is, an amount capable of eliciting an immune response in a subject to which the composition is administered. In the treatment and prevention of mastitis, for example, a "therapeutically effective amount" would preferably be an amount that enhances resistance of the mammary gland to new infection and/or reduces the clinical severity of the disease. Such protection will be demonstrated by either a reduction or lack of symptoms normally displayed by an infected host, a quicker recovery time and/or a lowered somatic cell count in milk from the infected quarter. For example, the ability of the composition to retain or bring the somatic cell count (SCC) in milk below about 500,000 cells per ml, the threshold value set by the International Dairy Federation, above which, animals are considered to have clinical mastitis, will be indicative of a therapeutic effect.

The exact amount is readily determined by one skilled in the art using standard tests. The LF-binding protein concentration will typically range from about 1% to about 95% (w/w) of the composition, or even higher or lower if appropriate. With the present vaccine formulations, 20 to 500 μg of active ingredient per ml of injected solution should be adequate to raise an immunological response when a dose of 1 to 3 ml per animal is administered.

To immunize a subject, the vaccine is generally administered parenterally, usually by intramuscular injection. Other modes of administration, however, such as subcutaneous, intraperitoneal and intravenous injection, are also acceptable. The quantity to be administered depends on the animal to be treated, the capacity of the animal's immune system to synthesize antibodies, and the degree of protection desired. Effective dosages can be readily established by one of ordinary skill in the art through routine trials establishing dose response curves. The subject is immunized by administration of the vaccine in at least one dose, and preferably two doses. Moreover, the animal may be administered as many doses as is required to maintain a state of immunity to infection.

Additional vaccine formulations which are suitable for other modes of administration include suppositories and, in some cases, aerosol, intranasal, oral formulations, and sustained release formulations. For suppositories, the vehicle composition will include traditional binders and carriers, such as, polyalkaline glycols, or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient in the range of about 0.5% to about 10% (w/w), preferably about 1% to about 2%. Oral vehicles include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium, stearate, sodium saccharin cellulose, magnesium carbonate, and the like. These oral vaccine compositions may be taken in the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations, or powders, and contain from about 10% to about 95% of the active ingredient, preferably about 25% to about 70%.

Intranasal formulations will usually include vehicles that neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known substances can be employed with the subject invention. The nasal formulations may also contain preservatives such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

Controlled or sustained release formulations are made by incorporating the protein into carriers or vehicles such as liposomes, nonresorbable impermeable polymers such as ethylenevinyl acetate copolymers and Hytrel® copolymers, swellable polymers such as hydrogels, or resorbable polymers such as collagen and certain polyacids or polyesters such as those used to make resorbable sutures. The LF-binding proteins can also be delivered using implanted mini-pumps, well known in the art.

The LF-binding proteins of the instant invention can also be administered via a carrier virus which expresses the same. Carrier viruses which will find use with the instant invention include but are not limited to the vaccinia and other pox viruses, adenovirus, and herpes virus. By way of example, vaccinia virus recombinants expressing the novel proteins can be constructed as follows. The DNA encoding the particular protein is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the instant protein into the

viral genome. The resulting TK⁻ recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

An alternative route of administration involves gene therapy or nucleic acid immunization. Thus, nucleotide sequences (and accompanying regulatory elements) encoding the subject LF-binding proteins can be administered directly to a subject for *in vivo* translation thereof. Alternatively, gene transfer can be accomplished by transfecting the subject's cells or tissues *ex vivo* and reintroducing the transformed material into the host. DNA can be directly introduced into the host organism, i.e., by injection (see International Publication No. WO/90/11092; and Wolff et al. (1990) *Science* 247:1465-1468). Liposome-mediated gene transfer can also be accomplished using known methods. See, e.g., Hazinski et al. (1991) *Am. J. Respir. Cell Mol. Biol.* 4:206-209; Brigham et al. (1989) *Am. J. Med. Sci.* 298:278-281; Canonico et al. (1991) *Clin. Res.* 39:219A; and Nabel et al. (1990) *Science* 249:1285-1288. Targeting agents, such as antibodies directed against surface antigens expressed on specific cell types, can be covalently conjugated to the liposomal surface so that the nucleic acid can be delivered to specific tissues and cells susceptible to infection.

Diagnostic Assays

As explained above, the LF-binding proteins of the present invention may also be used as diagnostics to detect the presence of reactive antibodies of *S. uberis* in a biological sample in order to determine the presence of *S. uberis* infection. For example, the presence of antibodies reactive with LF-binding proteins can be detected

using standard electrophoretic and immunodiagnostic techniques, including immunoassays such as competition, direct reaction, or sandwich type assays. Such assays include, but are not limited to, Western blots; agglutination tests; enzyme-labeled and mediated immunoassays, such as ELISAs; biotin/avidin type assays; radioimmunoassays; immunoelectrophoresis; immunoprecipitation, etc. The reactions generally include revealing labels such as fluorescent, chemiluminescent, radioactive, enzymatic labels or dye molecules, or other methods for detecting the formation of a complex between the antigen and the antibody or antibodies reacted therewith.

The aforementioned assays generally involve separation of unbound antibody in a liquid phase from a solid phase support to which antigen-antibody complexes are bound. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (e.g., in membrane or microtiter well form); polyvinylchloride (e.g., sheets or microtiter wells); polystyrene latex (e.g., beads or microtiter plates); polyvinylidine fluoride; diazotized paper; nylon membranes; activated beads, magnetically responsive beads, and the like.

Typically, a solid support is first reacted with a solid phase component (e.g., one or more LF-binding proteins) under suitable binding conditions such that the component is sufficiently immobilized to the support. Sometimes, immobilization of the antigen to the support can be enhanced by first coupling the antigen to a protein with better binding properties. Suitable coupling proteins include, but are not limited to, macromolecules such as serum albumins including bovine serum albumin (BSA), keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and other proteins well known to those

skilled in the art. Other molecules that can be used to bind the antigens to the support include polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and the like. Such molecules and methods of coupling these molecules to the antigens, are well known to those of ordinary skill in the art. See, e.g., Brinkley, M.A. *Bioconjugate Chem.* (1992) 3:2-13; Hashida et al., *J. Appl. Biochem.* (1984) 6:56-63; and Anjaneyulu and Staros, *International J. of Peptide and Protein Res.* (1987) 30:117-124.

After reacting the solid support with the solid phase component, any non-immobilized solid-phase components are removed from the support by washing, and the support-bound component is then contacted with a biological sample suspected of containing ligand moieties (e.g., antibodies toward the immobilized antigens) under suitable binding conditions. After washing to remove any non-bound ligand, a secondary binder moiety is added under suitable binding conditions, wherein the secondary binder is capable of associating selectively with the bound ligand. The presence of the secondary binder can then be detected using techniques well known in the art.

More particularly, an ELISA method can be used, wherein the wells of a microtiter plate are coated with an LF-binding protein. A biological sample containing or suspected of containing anti-LF-binding protein immunoglobulin molecules is then added to the coated wells. After a period of incubation sufficient to allow antibody binding to the immobilized antigen, the plate(s) can be washed to remove unbound moieties and a detectably labeled secondary binding molecule added. The secondary binding molecule is allowed to react with any captured sample antibodies, the plate washed and the presence

of the secondary binding molecule detected using methods well known in the art.

Thus, in one particular embodiment, the presence of bound anti-LF-binding antigen ligands from a biological sample can be readily detected using a secondary binder comprising an antibody directed against the antibody ligands. A number of anti-bovine immunoglobulin (Ig) molecules are known in the art which can be readily conjugated to a detectable enzyme label, such as horseradish peroxidase, alkaline phosphatase or urease, using methods known to those of skill in the art. An appropriate enzyme substrate is then used to generate a detectable signal. In other related embodiments, competitive-type ELISA techniques can be practiced using methods known to those skilled in the art.

Assays can also be conducted in solution, such that the LF-binding proteins and antibodies specific for those proteins form complexes under precipitating conditions. In one particular embodiment, LF-binding proteins can be attached to a solid phase particle (e.g., an agarose bead or the like) using coupling techniques known in the art, such as by direct chemical or indirect coupling. The antigen-coated particle is then contacted under suitable binding conditions with a biological sample suspected of containing antibodies for the LF-binding proteins. Cross-linking between bound antibodies causes the formation of particle-antigen-antibody complex aggregates which can be precipitated and separated from the sample using washing and/or centrifugation. The reaction mixture can be analyzed to determine the presence or absence of antibody-antigen complexes using any of a number of standard methods, such as those immunodiagnostic methods described above.

In yet a further embodiment, an immunoaffinity matrix can be provided, wherein a polyclonal population of antibodies from a biological sample suspected of containing anti-LF-binding molecules is immobilized to a substrate. In this regard, an initial affinity purification of the sample can be carried out using immobilized antigens. The resultant sample preparation will thus only contain anti-*S. uberis* moieties, avoiding potential nonspecific binding properties in the affinity support. A number of methods of immobilizing immunoglobulins (either intact or in specific fragments) at high yield and good retention of antigen binding activity are known in the art. Not being limited by any particular method, immobilized protein A or protein G can be used to immobilize immunoglobulins.

Accordingly, once the immunoglobulin molecules have been immobilized to provide an immunoaffinity matrix, labeled LF-binding proteins are contacted with the bound antibodies under suitable binding conditions. After any non-specifically bound antigen has been washed from the immunoaffinity support, the presence of bound antigen can be determined by assaying for label using methods known in the art.

Additionally, antibodies raised to the LF-binding proteins, rather than the LF-binding proteins themselves, can be used in the above-described assays in order to detect the presence of antibodies to the proteins in a given sample. These assays are performed essentially as described above and are well known to those of skill in the art.

The above-described assay reagents, including the LF-binding proteins, or antibodies thereto, can be provided in kits, with suitable

instructions and other necessary reagents, in order to conduct immunoassays as described above. The kit can also contain, depending on the particular immunoassay used, suitable labels and other packaged reagents and materials (i.e. wash buffers and the like). Standard immunoassays, such as those described above, can be conducted using these kits.

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

C. Experimental

Example 1

Identification of a Bovine Lactoferrin-Binding Protein in *S. Uberis*

Materials and Methods

Bacterial strains and cultural conditions.

S. uberis strain (su-1) from the American Type Culture Collection (ATCC 9927) was used for study. Bacteria were grown on base #2 sheep blood agar plates (PML Microbiologicals) at 37°C for 18 h. Iron-restricted conditions were achieved in Todd-Hewitt broth supplemented with 0.3% yeast extract (THB-YE) by the addition of 800 μM EDDA, 800 μM dipyridyl or 100 μM desferrioxamine mesylate. All of the iron chelators were obtained from Sigma.

Preparation of bacterial cell wall.

Cell wall components of *S. uberis* were extracted as described by Baker et al. (1976) *J. Exp. Med.* 143:258-270. Twenty base #2 blood agar plates were inoculated with *S. uberis* and incubated for 18 h

at 37°C. Bacterial cells were collected, washed once with 200 ml 0.85% saline and resuspended in 50 ml of extraction buffer (0.05 M Na_2HPO_4, 0.15 M NaCl, 0.01 M EDTA, pH 7.4). Cell walls were extracted by shaking with glass beads (4-mm diameter) for 20 h at 37°C. After centrifugation at 48,300 g for 20 min, supernatant (cell wall extract) was collected, filtered (0.22 μM Nalgene filter), dialysed against distilled water, lyophilized and resuspended in 1 ml of distilled water.

Preparation of iron-binding proteins.

All iron-binding proteins, including bLf (from bovine milk), bovine transferrin (bTf), human lactoferrin (hLf) and human transferrin (hTf), were purchased from Sigma in the most iron-free form available. Iron-saturated proteins and the apoproteins were prepared by methods described previously (Mazurier and Spik (1980) *Biochim. Biophys. Acta* 629:399-408).

Preparation of ^{125}I-labelled bLF.

Bovine Lf was iodinated by the lactoperoxidase method of Thorell and Johansson (1971) *Biochim. Biophys. Acta* 251:363-368. Approximately 70 μg of bLf (33% iron-saturated) was used for iodination; ^{125}I-labelled protein was separated from free $Na^{125}I$ by chromatography on a Sephadex G-25 column. The labelled protein was aliquoted and stored at -70°C until use. Lactoperoxidase was purchased from Boehringer-Mannheim; and $Na^{125}I$ from Amersham.

Lactoferrin-binding assays.

The binding assays were performed as described (Naidu et al. (1990) *J. Clin. Microbiol.* 28:2312-2319; Naidu et al. (1991) *J. Med. Micrbiol.*

34:323-328; Naidu et al. (1991) *J. Dairy Sci.* 74:1218-1226; Naidu et al. (1992) *J. Med. Microbiol.* 36:177-183). Bacterial cells were harvested from culture media, washed once in 0.1 M phosphate-buffered saline (PBS), pH 7.2, and resuspended in PBS containing 1% bovine serum albumin (PBS-1% BSA) to a density of 10^{10} bacteria/ml (c. OD_{600}=1.5). To determine saturation time, 10^9 bacteria (in 0.1 ml of PBS-1% BSA) were mixed and incubated with 0.1 ml of ^{125}I-bLf solution (6.9 nM in PBS-1 % BSA) for periods of 5, 10, 15, 20, 25, 30, 60, 90, 120, 150 min at room temperature. Bacteria were pelleted and washed three times with 1 ml of ice-cold PBS containing 0.1% Tween 20. Radioactivity bound to the bacterial pellet was measured in a γ-counter. In competitive binding experiments, 10^9 bacteria were mixed with 2 x 10^5 cpm ^{125}I-bLf in the presence of serially-diluted unlabelled bLf and incubated at room temperature for 2 h. Total input, cell bound and free proteins were calculated and subjected to Scatchard analysis (Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* 51:660-672). When evaluating the inhibitory effect of bLf, apo-bLf, bTf, hLF and hTf on ^{125}I-bLf binding, the unlabelled proteins were used at concentration of 5.5 μM. All samples were tested in triplicate, and each experiment was repeated at least twice. The data presented are the means of two independent experiments (unless otherwise stated).

Proteolytic and heat treatment of *S. uberis*.

Bacteria (1 ml containing 10^{10} cells) were treated with proteases at 37°C for 2 h. Trypsin (Sigma) hydrolysis was performed in 100 mM Tris-HCl (pH 8.0), with a final enzyme concentration of 2,500 u/ml, and the reaction was stopped by addition of phenylmethylsulfonyl fluoride (500 μg/ml). Pepsin (Sigma) digestion was performed in 100 mM sodium

acetate buffer (pH 4.5), with an enzyme concentration of 1,000 u/ml, and the pH of the reaction mixture was raised to 7.4 to stop the hydrolysis. Proteinase K (Boehringer mannheim) treatment was carried out in 40 mM potassium phosphate buffer (pH 7.5), and the digestion was inhibited by the addition of phenylmethylsulfonyl fluoride (500 μg/ml). For heat treatment, the bacterial suspension (10^{10} cell/ml) was incubated in a water bath for 1 h at each of the following temperatures: 50°C, 80°C and 100°C. Both enzyme and heat-treated cells were washed once in PBS and resuspended in PBS-1% BSA prior to the binding experiments.

PAGE and Western blotting.

SDS-polyacrylamide gel electrophoresis (PAGE) of proteins was performed using the method described by Laemmli (Laemmli, U.K. (1970) *Nature* 227:680-685). Samples were solubilized in sample buffer at 37°C for 30 min in the absence of 2-mercaptoethanol (non-reducing conditions) or at 100°C for 5 min in the presence of 1% 2-mercaptoethanol (reducing conditions). Proteins were electrophoretically transferred to nitrocellulose membranes as recommended by the supplier (Bio-Rad) and blocked with TBS-1% BSA.

To identify the putative bLf-binding protein, Western blots were probed with ^{125}I-bLf as follows. ^{125}I-bLf was added to the membrane to a final concentration of 80 ng/ml in TBS-1% BSA and incubated at room temperature for 2 h. After three washes with TBS containing 0.05% Tween 20, the membrane was exposed to X-ray film for 24 h at room temperature. To compete the ^{125}I-bLf binding, the transferred membrane was incubated with 35 μg/ml of unlabelled bLf for 2 h before incubation with ^{125}I-bLf.

Results

Time-dependent binding of bLF to *S. uberis*.

S. uberis strain su-1 was tested for bLf binding in a ^{125}I-labelled protein binding assay. To study the kinetics of ^{125}I-bLf binding with *S. uberis*, binding was measured at different time intervals (Figure 7). The time course showed that ^{125}I-bLf could bind to *S. uberis* in a time-dependent manner, with a requirement of approximately 90 min for 100% saturation. This binding saturation time was the basis for determining the incubation time in later binding experiments.

BLf receptor saturability, affinity and copy number.

A competitive binding experiment using bLf (33% iron-saturated) as both radioligand and competitor was performed, and the specificity of bLf binding by *S. uberis* was demonstrated (Figure 8). Unlabelled bLf effectively displaced the binding of ^{125}I-bLf to *S. uberis* in a dose-dependent manner. A concentration of approximately 270 nM of unlabelled bLf caused 50% blocking of ^{125}I-bLf uptake (indicated by dotted lines). Scatchard plot analysis showed linearity, thus the demonstration of one bLf binding component is expected. The number of bLf molecules bound per *S. uberis* cell calculated from Scatchard plot was approximately 7800, with an affinity (Kd) of 1.0×10^{-7}M.

To determine whether bLf iron saturation could influence receptor binding, apo-bLf was used as a competitor in the ^{125}I-bLf binding assay. The results showed that apo-bLf could inhibit ^{125}I-bLF-binding as effectively as the iron-saturated bLf (Table 1), indicating that both apo-bLf and iron-saturated bLf had the same binding receptor on the *S. uberis* cell.

To examine further the specificity of binding, the abilities of bTf, hLf and hTf to inhibit the binding of ^{125}I-bLf to *S. uberis* cells were also evaluated (Table 1). None of these proteins interfered with the binding of ^{125}I-bLf to *S. uberis*, suggesting that the binding was bLf-specific.

Influence of iron-restricted conditions on bLf binding.

In an attempt to determine if the bLf-binding property of *S. uberis* was mediated by an iron-regulated bacterial component, EDDA, dipyridyl or desferrioxamine mesylate were incorporated in THB-YE broth to reduce the availability of iron. Cells from these iron-restricted conditions did not show higher ^{125}I-bLf binding than those from normal cultural condition (Figure 9), indicating that iron-restricted condition did not notably modify the saturation of *S. uberis* by bLf.

Table 1. Inhibitory effects of unlabelled proteins and enzyme or heat treatment of bacteria on ^{125}I-bLf binding to *S. uberis*.

Inhibitor or cell treatment	Percentage inhibition[1] or decrease[2] in binding	
	mean	SD
Inhibitor		
bLf	94.6	0.1
apo-bLf	94.7	0.2
bTf	2.5	1.4
hLf	-14.0	2.4
hTf	1.9	1.3
Proteases		
Pepsin	85.7	3.7
Trypsin	89.1	4.4
Proteinase K	92.7	2.4
Heating		
50°C	-4.3	6.0
80°C	29.6	2.1
100°C	65.6	4.8

[1] Inhibition values were calculated as relative percentage of bLf binding to bacteria suspended in PBS in the absence of any inhibitor.
[2] Decrease in binding were calculated as relative percentage of bLf binding to bacteria without any treatment.

Sensitivity of the bLf binding component to protease hydrolysis and heat treatment.

Pepsin, trypsin and proteinase k treatment of *S. uberis* cells could abolish bLf-binding (Table

1), indicating the involvement of a surface-exposed cell wall protein(s) in the binding. This proteinaceous component was susceptible to temperature, since heat treatment of bacteria reduced binding to a certain degree (Table 1).

Identification of a cell wall bLf-binding protein.

The presence of the functionally active bLf-binding protein (Lbp) in the cell wall preparation of *S. uberis* was detected by a Western blot probed with ^{125}I-bLf. Under non-reducing conditions, two components with apparent molecular weights of 165 and 76 kDa, respectively, were identified as bLf-binding proteins of *S. uberis*. Proteins under reducing conditions lost the bLf binding activity to a great extent. The protein bands were demonstrated to be from specific binding to ^{125}I-bLf, since the presence of unlabelled bLf effectively blocked the binding.

Discussion

To demonstrate whether *S. uberis* was able to express a specific receptor for bLf, the inventors herein sought to determine whether the major prerequisites for a biological receptor could be fulfilled, namely, ligand specificity and concentration-dependent saturability. Bovine Lf binding to *S. uberis* was time and concentration dependent (demonstrated by the ability of the unlabelled ligand to compete for binding with ^{125}I-bLf), indicating the existence of a limited number of binding receptors on the cell surface. Scatchard plot analysis estimated that there were 7800 bLf-binding sites/cell. The affinity of the streptococcal receptor for bLf (1.0×10^{-7} M) is slightly lower than that described by Naidu et al. (1991) *J. Dairy Sci.*

74:1218-1226 for the bLf receptor of *S. aureus* (7.1×10^{-8} M).

S. uberis did not distinguish between bovine apo-Lf and iron-saturated Lf, since both forms were equally effective at blocking ^{125}I-bLf binding in the competitive binding assay. This finding is similar to that described for the *N. meningitidis* Lf receptor (Schryvers and Morris (1988) *Infect. Immun.* 56:1144-1149) but contrasts with that for the Tf receptors.

A common feature which has emerged from studies of the transferrin-binding proteins of Gram-negative bacteria is their remarkable specificity for the transferrin of their natural host. In competition binding assays, the streptococcal receptor described here also demonstrated some lactoferrin species specificity in that human Lf could not effectively block the binding of bovine Lf. Interestingly, *S. aureus* Lf receptors have been shown to bind lactoferrins from both human and bovine sources (Naidu et al. (1990) *J. Clin. Microbiol.* 28:2312-2319; Naidu et al. (1991) *J. Med. Micrbiol.* 34:323-328; Naidu et al. (1991) *J. Dairy Sci.* 74:1218-1226; Naidu et al. (1992) *J. Med. Microbiol.* 36:177-183). In this context, given that *S. uberis* is exclusively a bovine pathogen, while *S. aureus* causes infection in both humans and bovine, it is possible that the specificity for lactoferrins may also contribute to the host specificity of these bacteria. In addition, neither bovine nor human transferrin was able to block binding of ^{125}I-labelled bovine lactoferrin to the lactoferrin receptor of *S. uberis*. This observation is similar to that reported by Naidu et al. (1991) *J. Dairy Sci.* 74:1218-1226; Naidu et al. (1992) *J. Med. Microbiol.* 36:177-183, in which human or bovine lactoferrin-binding to lactoferrin receptors of *S. aureus* could not be blocked by human or bovine transferrin.

Although the expression of most of the transferrin and lactoferrin receptors of Gram-negative bacteria such as *N. meningitidis* and *H. influenza* is iron regulated, *S. uberis* receptor activity was not regulated by growth medium iron availability. The results are consistent with the findings of Modun et al. (1994) *Infect. Immun.* 62:3850-3858 and Rainard, P. (1992) *FEMS Microbiol. Lett.* 98:235-240 who showed that the binding of *S. aureus* to Tf and *S. agalactiae* to Lf was not regulated by iron.

Having demonstrated the existence of bovine lactoferrin-receptor on *S. uberis*, the inventors herein sought to identify the bacterial cell wall component(s) involved. Treatment of *S. uberis* cells with heat and proteolytic enzymes abolished lactoferrin-binding, indicating the probable involvement of a protein(s) on the cell surface. A cell wall extract of *S. uberis* was prepared and solubilized in SDS-PAGE sample buffer under non-reducing condition. Two lactoferrin-binding proteins with molecular weights of 165 kDa and 76 kDa were identified by ^{125}I-bLf binding after SDS-PAGE and transfer to nitrocellulose. Since Scatchard plot analysis indicated that *S. uberis* bears only one bLf binding component, the 165 kDa protein is likely the dimer form of the 76 kDa.

The lactoferrin-binding protein of *S. uberis* differs from the transferrin receptors of *Haemophilus* and *Neisseria* spp., which consist of two distinct transferrin-binding proteins, termed Tbp1 and Tbp2, which range in molecular weight from 68 to 105 kDa depending on the strain. The *S. uberis* Lbp is also different from the lactoferrin receptors of the mentioned two species. To date, only one lactoferrin-binding protein with a molecular weight of 98 to 105 kDa has been identified from those species above and

the gonococcal lactoferrin protein (Lbp1) has been shown to share features with the functionally related gonococcal Tbp1 (Biswas and Sparling, 1995). The bovine lactoferrin receptor of *S. aureus* consists of two distinct bLf-binding proteins with estimated molecular weights of 92 and 67 kDa (Naidu et al. (1991) *J. Dairy Sci.* 74:1218-1226) and therefore appears to be different than the receptor described herein. Also, the streptococcal Lbp described here appears to be different from the *S. aureus* human lactoferrin-binding protein which is as an approximately 450 kDa protein which under reducing SDS-PAGE gel conditions resolves into two components of 67 and 62 kDa.

Example 2

Cloning and Characterization of *S. uberis* Lbp Gene and its Upstream Mga

Materials and Methods

Bacterial strains, plasmids and media.

S. uberis strains used are listed in Table 2 below. Bacteria were grown on base #2 sheep blood agar plates (PML Microbiologicals) at 37°C for 18 h, or in Todd-Hewitt broth supplemented with 0.3% yeast extract (THB-YE) at 37°C overnight. *E. coli* cells were grown in Luria broth or on Luria broth-agar plates. Ampicillin was used at 50 μg/ml for the growth of *E. coli* strains containing recombinant plasmids. The cloning vector used was pTZ18R (Mead et al. (1986) *Protein Eng.* 1:67-74).

Preparation of bacterial cell wall, cell membranes, periplasma, whole cell lysate and culture supernatant.

Cell wall components of *S. uberis* were extracted as described in Example 1. Outer and inner

membranes were isolated from *E. coli* cells by sucrose density gradient centrifugation. *E. coli* periplasmic proteins were prepared by the cold osmotic shock method.

To prepare whole cell lysates of *E. coli* transformants, bacteria were grown overnight, collected, washed once in 0.1 M phosphate-buffered saline (PBS), pH 7.2, resuspended in water, exposed to one freeze-thaw cycle and sonicated for 2 min. After centrifugation at 6000 x g for 20 min, the supernatant (whole cell lysate) was collected, lyophilized and resuspended in 1/100 culture volume of water.

The 100 fold-concentrated culture supernatant of the recombinant *E. coli* was obtained by precipitation with 10% trichloroacetic acid (TCA).

Preparation of iron-saturated bLf and ^{125}I-labelled bLf.

33% iron-saturated bovine lactoferrin was prepared and iodinated as described in Example 1.

Lactoferrin-binding assay.

Bacterial cells were harvested from culture media, washed once in 0.1 M PBS (pH 7.2), and resuspended in PBS containing 1% bovine serum albumin (PBS-1% BSA) to a density of 10^{10} bacteria/ml (c. OD_{600}=1.5). To assess the bLf-binding ability of the recombinant protein, increasing amounts of *E. coli* cell lysate plus supernatant were mixed with approximately 10^9 *S. uberis* and incubated with 1.0 x 10^4 cpm ^{125}I-bLf in a total volume of 200 µl. After 2 hours of incubation at room temperature, bacterial cells were pelleted and washed three times with 1 ml of ice-cold PBS containing 0.1 % Tween 20. Radioactivity present in the bacterial pellet was measured in a gamma-counter. All samples were tested

in triplicate, and the experiment was repeated twice. The data presented are the mean of two independent experiments ± standard deviation.

Preparation of antiserum.

Serum against the recombinant Lbp of *S. uberis* was raised in rabbits by subcutaneous injection of 0.5 ml of TCA-precipitated culture supernatant of recombinant *E. coli* in complete Freund's adjuvant. Two subcutaneous boosts with the same amount of sample in incomplete Freund's adjuvant were given to each animal.

PAGE and Western blotting.

SDS-polyacrylamide gel electrophoresis (PAGE) of proteins was performed as described by Laemmli (Laemmli, U.K. (1970) *Nature* 227:680-685). Samples under reducing and non-reducing conditions were prepared as described in Example 1. Samples dissolved in sample buffer with 3 M urea were boiled for 30 min prior to loading. ^{125}I-bLf probed-Western blotting was performed as in Example 1. Immunoblotting probed with rabbit antiserum against the recombinant Lbp was performed as follows. Proteins were electroblotted onto nitrocellulose membranes as recommended by the supplier (Bio-Rad). Nonspecific binding was blocked by incubation in TBS (10 mM Tris-HCl, pH 7.5, 140 mM NaCl)-1% bovine serum albumin (BSA). Blots were incubated with antibody diluted 1:200 in TBS-1% BSA at room temperature for 1 h. After three washes in TBS containing 0.05% Tween 20, seroreactive proteins were detected with goat anti-mouse (or rabbit) IgG coupled to alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc.) at 1:5,000 in TBS-1% BSA. Alkaline phosphatase activity was detected using the Nitro Blue Tetrazolium-5-bromo-

4-chloro-3-indolylphosphate toluidinium system as described by the supplier (Promega).

Recombinant DNA techniques.

Plasmid DNA was purified as described by Sambrook et al., *supra*. When required, DNA fragments were isolated from agarose gels using a Gene Clean kit (Bio/can Scientific).

To construct a gene library for *S. uberis*, chromosomal DNA was prepared as previously described (Caparon and Scott (1987) *Proc. Natl. Acad. Sci. USA* 84:8677-8681) and partially digested with *Sau*3AI. Fragments of 2,000 to 5,000 bp were recovered following sucrose density gradient centrifugation (Sambrook et al., *supra*). The ends of these fragments were partially filled in with dGTP and dATP and ligated into pTZ18R which was cut with *Sal*I and partially filled in with dTTP and dCTP. Transformation of *E. coli* DH5α competent cells was carried out as recommended by the supplier (GIBCO BRL, Gaithersburg, Md). To identify Lf-binding clones, transformants were replica-plated onto nitrocellulose discs (Schleicher & Schuell, Keene, NH) and lysed in chloroform vapor. Nonspecific binding was blocked by incubation with TBS-1% BSA. Membranes were further incubated with ^{125}I-bLf as described in Example 1.

Restriction endonucleases, T4 DNA ligase, DNA polymerase I Klenow fragment and calf intestinal alkaline phosphatase were utilized according to the manufacturer's directions (Pharmacia Canada Ltd., Quebec, Canada).

DNA sequences were determined by the dideoxy-chain termination method of Sanger et al. (1977) *Proc. Natl. Acad. Sci. USA* 74:5463-5467 on double-stranded plasmid templates by using a T7 Sequencing kit (Pharmacia Canada Ltd.).

RNA analyses.

RNA from *E. coli* strains was isolated as described previously (Lloubes et al. (1986) *Nucleic Acid Res.* 14:2621-2636) with an additional RNase-free DNase I digestion.

RNA from *S. uberis* was prepared as follows. The cell pellet from a 10 ml culture (OD_{600}=0.6) was resuspended in 250 µl of TE buffer (pH 8.0) containing 500 u of mutanolysin (Sigma) and incubated at 37°C for 30 min. Lysis buffer (250 µl) (60 mM Tris-HCl pH 7.4, 200 mM NaCl, 10 mM EDTA, 2% SDS) and 100 µg/ml (final concentration) of proteinase K was added and the incubation continued for 1 h. The sample was extracted once with 65°C phenol (water saturated, pH 4.0) and twice with room temperature phenol. RNA was recovered by ethanol precipitation and treated with DNase I (Pharmacia Canada Ltd.).

Northern blot analysis was carried out as described by Sambrook et al., *supra*.

Results

Cloning and expression of the *lbp* gene.

A gene library was constructed in pTZ18R with chromosomal DNA from *S. uberis* (su-1). About 5000 transformants were initially screened for expression of bLf binding protein (Lbp) by colony blotting with ^{125}I-bLf. One colony with the strongest signal and six with weaker signals were selected and used to make whole cell lysates, which were further tested for their ability to bind ^{125}I-bLF under non-reducing conditions. The clone with the strongest signal, *E. coli* pLBP5, generated three major bands; two of them had molecular weights of 165 and 76 kDa which are quite close in size to those of *S. uberis*. A band slightly larger than 165 kDa was also observed which could be the precursor form with the uncleaved

signal peptide. No corresponding band was found from the whole cell lysate of *E. coli* pTZ18R, the host strain control. The recombinant Lbp was also detected from the periplasm and supernatant of *E. coli* pLBP5, but not from outer or inner membranes, indicating that the Lbp expressed in *E. coli* DH5α was not membrane-localized, but instead could be secreted from the cell.

The presence of free and functionally active recombinant Lbp in the cell lysate and supernatant of *E. coli* pLBP5 was also detected by performing competitive inhibition assays. The *E. coli* pLBP5 cell lysate and supernatant mixture effectively inhibited ^{125}I-bLf binding to *S. uberis* cells in a dose-dependent manner, while samples from *E. coli* pTZ18R did not (Figure 11).

To demonstrate whether the protein with a molecular weight of 165 kDa was a dimer of the 76 kDa molecule, samples treated with 2-mercaptoethanol or urea were analyzed by Western blotting using rabbit antiserum against the supernatant of *E. coli* pLBP5. Mercaptoethanol treatment was shown to have no effect on the electrophoretic mobility of Lbp from either *S. uberis* or recombinant *E. coli* while urea-treatment resulted in disappearance of the 165 and 76 kDa bands and appearance of a new band with an apparent molecular weight of 105 kDa. This data indicates that the 165 kDa protein is likely a dimer of the 76 kDa subunit. The dimer could have been disassociated by urea to monomers which could be further denatured and unfolded, resulting in the apparent molecular weight increase from 76 kDa to 105 kDa. A significant increase in the apparent molecular weight of Tbp2 following urea-treatment has been observed in *Neisseria meningitidis* by other researchers (Vonder haar et al. (1994) *J. Bacteriol.* 176:6207-6213). The

above data also indicates that disulfide bonds are not essential for the formation of the oligomer.

Nucleotide sequence determination and analysis.

To determine the nucleotide sequence of the Lbp gene, each of the *Hinc*II, *Hind*III, *Sac*I, *Sph*I and *Xba*I fragments of pLBP5 (Figure 1) was individually cloned into the corresponding site of pTZ18R. By using universal and reverse primers, each fragment was sequenced in both orientations. Subsequent primers were synthesized on the basis of sequence information thereby obtained.

Two open reading frames were found from pLBP5 (Figure 1). One was the Lbp-encoding gene *lbp*, whose presence in subclone pLBP5L resulted in a bLf-binding phenotype. The other ORF on the complementary DNA strand was incomplete. A GenBank database search showed that the presumed ORF gene product had significant homology to the VirR and Mry positive regulators in group A streptococci. Thus, this ORF was named *mga'* (Figure 1). The cloning and sequencing of the complete *mga* gene is discussed further below.

The *lbp* sequence contained two potential translation start codons (ATG) at positions 232 and 262 of the DNA sequence. Both are associated with putative Shine-Dalgarno sequences (Figures 2A-2C). These start points would give proteins with predicted sizes of 62.857 and 61.454, respectively. The predicted sizes are comparable to 76 KDa molecular weight protein. The reason for the discrepancy between the observed and calculated molecular weights of this protein is not clear. Posttranslational modification, such as lipid modification might have occurred and increased the apparent molecular mass in the SDS-PAGE determination. A similar difference observed in Gram-negative bacteria has been

demonstrated to be caused by protein lipid modification (Theisen et al. (1992) *Infect. Immun.* 60:826-831; Theisen et al. (1993) *Infect. Immun.* 61:1793-1798).

The DNA sequence shows two putative -10 and -35 promoter regions present at -88 and -102 from the first ATG. Downstream of *lbp* there is a potential rho-independent transcription terminator (Figures 2A-2C).

Analysis of the N-terminus of the predicted sequence of Lbp showed amino acids characteristic of signal sequences (Simonen and Palva (1993) *Microbiol. Rev.* 57:109-137) (Figures 2A-2C). The features of the sequence are a positively charged N-terminus, rich in K and R residues, followed by a hydrophobic domain from amino acids 25 to 48 (Figure 3) and the signal peptidase cleaving site, VKA, at positions 49 to 50, where cleavage occurs after the A residue. The presence of this putative signal sequence indicates that the Lbp is exported across the cytoplasmic membrane of *S. uberis*.

A search of the GenBank database revealed that the C-terminus of the Lbp was highly homologous to the C- terminal ends of streptococcal M proteins, plasminogen binding protein, fibrinogen and IgG-binding proteins. It shows all the general features well established for surface proteins of Gram-positive cocci (Fischetti et al. (1991) Common characteristics of the surface proteins from Gram-positive cocci, p. 290-294. In G. M. Dunny, P. P. Cleary, and L. L. McKay (ed), *Genetics and molecular biology of streptococci, lactococci, and enterococci*. American Society for Microbiology, Washington, D. C.). These features include a small cluster of four charged amino acids at the C-terminus followed by a hydrophobic domain of 21 amino acids (Figures 2A-2C and Figure 3). Adjoining

the hydrophobic domain is the consensus membrane anchor motif LPSTGD. The next region of 50 amino acids is the cell wall-associated region characterized by a high proportion of proline and glycine (12%). GenBank database searches also showed that Lbp is homologous to mammalian myosin heavy chain and kinesin heavy chain; it has 48% and 46% overall sequence homology with these two fibrillar proteins, respectively.

The region beyond the membrane anchor motif and proline/glycine rich region contains three blocks of amino acids which were found to have internal homologies (Figures 2A-2C). The A1 block contains 52 amino acids homologous to the A2 block; the B1 block contains 13 amino acids homologous to the B2 block; and the 59 amino acid C1 block is homologous to the C2 block.

Analysis of the secondary structure of the translated protein showed an extensive α-helix region stretching from the proline/glycine-rich region to the β-sheet and turn region near the cleavage site of the signal sequence (Figure 3).

Localization of the Lf-binding domain of Lbp.

To localize the Lf-binding domains of Lbp, gene deletions were constructed (Figure 1). 3'-deletions, pTP31, pTP32, pTP33 and pTP34 were generated at the restriction enzyme sites *Hinc*II, *Stu*I, *Xba*I and *Xmn*I of pLBP5L. A 5'-deletion pTP51 was constructed by removing the 643 bp *Hind*III-*Xba*I fragment of pLBP5L. All these deletions contained the *lbp* promoter. The resulting truncated Lbps were produced in *E. coli* and were visualized after SDS-PAGE, transfer to nitrocellulose, and reaction with specific rabbit antiserum or ^{125}I-bLf. All the samples were tested under non-reducing conditions in these

experiments. The bLf binding results are summarized in Figure 1. Like full-length Lbp, truncated proteins from *E. coli* pTP31, pTP32 and pTP33 could bind bLf in both monomeric and dimeric forms. Some degradation products from these clones also possessed binding ability. No band was found from *E. coli* pTP34 or pTP51. These data indicate that the primary domain of Lbp involved in the binding of bLf resides in a 22 kDa N-terminal fragment.

Cloning and sequencing of the complete *mga* gene.

As described above, the plasmid pLBP5 contained an incomplete gene, *mga'*, in addition to *lbp*. The pLBP5 molecule was inverted to pLBP5i (Figure 4) for convenience of description.

To obtain the complete *mga* gene, a 991 bp *Sty*I fragment from *mga'* was radioactively (^{32}P) labelled as a probe (VP1 in Figure 4) and used to screen an *S. uberis* gene library. A positive *E. coli* DH5α clone was obtained and the restriction enzyme map of the plasmid pMGA14 is shown in Figure 4. To determine the nucleotide sequence of pMGA14, each of the *Bam*HI, *Hinc*II, *Hind*III and *Kpn*I restriction fragments of pMGA14 (Figure 4) was individually cloned into the corresponding site of pTZ18R and sequenced from both orientations using universal and reverse primers. Subsequent primers were synthesized on the basis of sequence information thereby obtained. Plasmid pMGA14 contained the complete ORF of Mga. However, the associated promoter region was not present. Since the 1.5 kb *Sph*I-*Nhe*I fragment of pLBP5i contained the majority of the 5' region of the *mga'* and the complete promoter region, it was inserted into the *Sph*I and *Nhe*I sites of pMGA14 to generate pMGA14F which then contained the complete *mga* gene including the promoter (Figure 4).

The sequence of a 3558 bp DNA fragment of pMGA14F is presented in Figures 5A-5D. Starting at the ATG initiation codon at nucleotides 361-363 and terminating at a TAA codon at nucleotides 1858-1860, the deduced gene product, Mga, is comprised of 499 amino acid residues with a calculated molecular weight of 58,454 Da. The N- terminus of Mga lacks the features of signal peptides as described (Simonen and Palva (1993) *Microbiol. Rev.* 57:109-137), suggesting that it is a cytoplasmic protein. Preceding the start codon of *mga* is a putative ribosome binding site AGGAGA. Sequences resembling the -35 and -10 promoter motifs have also been identified.

A search of the GenBank database revealed that the deduced protein of *mga* has 34% overall sequence identity to the VirR and Mry proteins (Chen et al. (1993) *Mol. Gen. Genet.* 241:685-693); Perez-Casal et al. (1991) *J. Bacteriol.* 173:2617-2624). Studies have shown that these positive regulators of group A streptococcal M proteins contain helix-turn-helix DNA binding domains, and it is believed that via these domains, the regulators may interact directly with specific DNA sequences to influence transcriptional activity (Chen et al., *supra*; Perez-Casal et al., *supra*). Attempts to identify a similar DNA-binding domain with visual inspection and analysis with PCGENE software failed to detect such a motif in Mga of *S. uberis*. However, a region at amino acid residues 106 to 125 (Figures 5A-5D) showed 90% identity to the sequence of the DNA-binding domain of VirR49 (Podbielski et al. (1995) *Infect. Immun.* 63:9-20). Following *mga*, there are four more ORFs with 181, 85, 78 and 128 amino acid residues, respectively (Figures 5A-5D). No significant sequence alignments were found from GenBank.

Northern blot analysis of the *lbp* and *mga* transcripts.

To analyze the *lbp* and *mga* transcripts, RNA was prepared from *S. uberis* (su-1), *E. coli* DH5α(pLBP5), *E. coli* DH5α(pLBP5L), *E. coli* DH5α(pMGA14F) and *E. coli* DH5α(pTZ18R), and used for two Northern blots. One blot was probed with the 1.5 kb *Hind*III-*Hpa*I internal fragment of *lbp* (LP1 in Figure 6). A 2.0 kb band was seen in lanes containing RNA from *S. uberis*, *E. coli* DH5α(pLBP5) and *E. coli* DH5α(pLBP5L), but was absent in the sample which contained RNA from *E. coli* DH5α(pTZ18R). This data indicates that only one major transcript was generated by *lbp* in recombinant *E. coli* as well as in native *S. uberis*. The second blot was probed with the 1.0 kb *Nco*I-*Nhe*I internal fragment of *mga* (VP2 in Fig. 4). A 1.8 kb band was seen in samples which contained RNA from *E. coli* DH5α(pLBP5) and *E. coli* DH5α(pMGA14F), respectively. No band was found from *S. uberis* and *E. coli* DH5α(pTZ18R) RNA samples. The *mga* and *mga'* genes were transcribed in recombinant *E. coli*, and the stop codon of the vector must have been utilized during the transcription of *mga'* gene. However, it is unclear whether the absence of a visible hybridization band from *S. uberis* was due to the low quantity of the gene transcript or the inactivity of the *mga* gene.

Southern blot analysis of the *lbp* and *mga* distribution in *S. uberis* strains.

A collection of *S. uberis* including five ATCC strains and 42 field isolates (Table 2) was used in Southern hybridization experiments. Chromosomal DNA was prepared and digested with the restriction endonuclease *Hind*III and separated on agarose gels. To analyze the *lbp* gene distribution, a 1.5 kb *Hind*III-*Hpa*I fragment which contained the most DNA sequence from the region encoding Lbp (LP1 in Figure

6) was used as a probe. This probe hybridized with DNA from 42/47 strains (Table 2). This result meant either that *lbp* shared a region of homology with most of the *S. uberis* strains or that among these strains, there were different regions of the *lbp* gene homologous to the *lbp* probe. To locate the homologous region(s) more specifically, this *lbp* probe was subdivided into smaller segments (Figure 6). Two probes, the 643 bp *Hin*dIII-*Xba*I fragment (LP2) and the 437 bp *Xba*I-*Stu*I fragment (LP3) covering the N-terminal and central portion of the coding region in the *lbp* gene, respectively, hybridized only with su-1, the strain from which the *lbp* gene was originally cloned, whereas the 409 bp *Stu*I-*Hap*I fragment from the C-terminal region (LP4) hybridized with all the strains that were hybridized by probe LP1 (Table 2). It appears to be clear that the region of the *lbp* gene encompassing the coding sequences for part of the C repeat, the proposed wall attachment region and the membrane anchor, is conserved among *S. uberis* strains, while the region encoding the N-terminal portion varies greatly. The diversity in sizes of chromosomal restriction fragments from the different strains that hybridized with the *lbp* probes indicates some degree of restriction site heterogeneity (a different location for *Hin*dIII site) among these strains.

Table 2. *S. uberis* strains and DNA hybridization with LP and VP probes

Strain	Source[a]	Probe				
		LP1	LP2	LP3	LP4	VP3
su-1	ATCC9927	3.40[b]	3.40	3.40	3.40	0.87
su-2	ATCC13386	-[c]	-	-	-	-
su-3	ATCC13387	3.60	-	-	3.60	3.60
su-4	ATCC19436	2.70	-	-	2.70	1.70
su-5	Chirino 93-1869	-	-	-	-	-
su-6	Chirino 93-2017	-	-	-	-	-
su-7	Chirino 93-8678-2	4.40	-	-	4.40	4.40
su-8	Chirino-*S. uberis*	5.00	-	-	5.00	0.91
su-9	ATCC27958	3.80	-	-	3.80	0.87
su-10	Greenfield 93-4997	3.20	-	-	3.20	1.50
su-11	Greenfield 93-4997	3.20	-	-	3.20	1.50

Table 2. (continued)

Strain	Source[a]	Probe				
		LP1	LP2	LP3	LP4	VP3
su-12	Leduc	3.80	-	-	3.80	0.87
su-13	Leduc	3.80	-	-	3.80	0.87
su-14	Alix	5.00	-	-	5.00	5.00
su-15	Magrath	4.10	-	-	4.10	0.90
su-16	Ohaton	5.70	-	-	5.70	0.90
su-17	Winfield	2.90	-	-	2.90	1.40
su-18	Not known	4.10	-	-	4.10	1.00
su-19	Gibbons	3.60	-	-	3.60	1.20
su-20	Ardrossan	4.40	-	-	4.40	4.40
su-21	Not known	3.40	-	-	3.40	1.00
su-22	Dapp	2.90	-	-	2.90	1.42
su-23	Thorsby	3.20	-	-	3.20	1.40
su-24	Ponoka	3.80	-	-	3.80	0.87
su-26	Warburg	4.40	-	-	4.40	0.90
su-27	Leduc	-	-	-	-	-
su-28	Fort MacLeod	3.00	-	-	3.00	1.80
su-29	Sherwood Park	5.00	-	-	5.00	5.00
su-30	Barrhead	5.00	-	-	5.00	5.00
su-31	Wainwright	5.00	-	-	5.00	5.00
su-32	Tofield	3.60	-	-	3.60	1.03
su-33	Barrhead	5.00	-	-	5.00	5.00
su-34	Barrhead	5.00	-	-	5.00	5.00
su-35	Medicine Hat	5.00	-	-	5.00	5.00
su-36	Ponoka	5.00	-	-	5.00	5.00
su-37	Ponoka	5.00	-	-	5.00	5.00
su-38	Ponoka	5.00	-	-	5.00	5.00
su-39	Ponoka	5.00	-	-	5.00	5.00
su-40	Millet	3.60	-	-	3.60	1.03
su-41	Lacombe	3.00	-	-	3.00	1.40
su-42	Daysland	5.00	-	-	5.00	5.00
su-43	Ohaton	5.30	-	-	5.30	0.97
su-44	Ohaton	5.00	-	-	5.00	5.00
su-45	Didsbury	-	-	-	-	-
su-46	Didsbury	4.70	-	-	4.70	4.70
su-47	Didsbury	5.00	-	-	5.00	5.00
su-48	Didsbury	4.70	-	-	4.70	4.70

[a] All strains are field isolates except five ATCC (American Type Culture Collection) strains.
[b] Size of hybridizing fragment (in kilobase).
[c] Lack of hybridization.

In order to study whether there was an *mga*-related gene in other *S. uberis* strains, the previous blot was stripped and reprobed with a 572 bp *Hin*dIII

fragment that encompassed the 5'-region of the *mga* gene (VP3 in Figure 4). Hybridization was detectable in all *S. uberis* strains that showed homology with the *lbp* gene (Table 2). The specific band which reacted with the *mga* probe in each strain is listed in Table 2.

Discussion

The Lbp gene of *S. uberis* was cloned in *E. coli* after screening a gene library by colony blotting with ^{125}I-bLf. *E. coli* transformants produced functionally active bLf-binding proteins with molecular weights of 76 kDa and 165 kDa, similar to the native proteins produced by *S. uberis*. Considering the one-affinity binding phenomenon of bLf with *S. uberis* (Example 1), we wondered whether the 165 kDa protein was a dimer of the 76 kDa molecule. Treatment with the reducing reagent β-mercaptoethanol did not have any effect on the mobilities of these proteins, indicating the absence of disulfide bridges in the protein structure. This was confirmed after the sequencing data was available; there are no appropriate cysteine residues within this protein. After boiling for 30 min in the presence of 3 M urea, proteins were completely denatured. This treatment resulted in a significant molecular weight decrease of the 165 kDa protein, probably resulting from the dissociation of the dimer, and an increase in molecular weight of the 76 kDa resulting from protein unfolding. The dimer structure seems to be very stable, since treatment with lower concentration of urea or shorter boiling time could not alter the mobility of the 165 kDa band. The presence of both monomeric and dimeric Lbp in *S. uberis* cell wall preparations may imply the existence of both forms on the bacterial surface. However, it is possible that

only the dimeric form exists on the bacterial cell; the monomer might come from the cytoplasm due to partial cell lysis caused by glass bead treatment. The dissociation of the dimer to a monomer during sample preparation seems unlikely because of the stability of the dimer.

The complex nature of the *S. uberis* Lbp was further confirmed from the nucleotide sequence analysis. As expected, the two Lbps were encoded by a single ORF of 1,683 bp. It is quite possible that two copies of the translated product interact with each other to form a homodimer. Interactions between subunits could be extensive and extend throughout the length of the molecule due to the existence of the high α-helical content. Like M proteins (Fischetti, V.A. (1989) *Clin. Microbiol. Rev.* 2:285-314), Lbp shares significant sequence homology with a number of α-helical coiled structure-containing mammalian fibrillar proteins such as human myosin heavy chain and kinesin heavy chain.

The deduced amino acid sequence of Lbp indicated the existence of a signal peptide of 50 amino acids (if translation started at the first ATG start codon) at the N-terminus, as expected for a protein that appears on the outside of a bacterial cell. The structure of this signal peptide is comparable to the consensus structures for signal peptides in proteins from Gram positive bacteria described previously (Simonen and Palva (1993) *Microbiol. Rev.* 57:109-137; Goward et al. (1993) *Trends Biochem. Sci.* 18:136-140; Perlman and Halvorson (1983) *J. Mol. Biol.* 167:391-409; von-Heijne, G. (1983) *Eur. J. Biochem.* 133:17-21; von-Heijne, G. (1986) *Nucleic Acids Res.* 14:4683-4690).

Analysis of the C-terminus of Lbp revealed the presence of an amino acid sequence typical of

membrane anchor motifs found in many other proteins from Gram- positive bacteria (Fischetti et al. (1991) Common characteristics of the surface proteins from Gram-positive cocci, p. 290-294. In G. M. Dunny, P. P. Cleary, and L. L. McKay (ed), *Genetics and molecular biology of streptococci, lactococci, and enterococci*. American Society for Microbiology, Washington, D. C.). Such regions have been shown to play a role in the anchoring of proteins in the cell wall (Pancholi and Fischetti (1988) *J. Bacteriol.* 170:2618-2624; Schneewind et al. (1992) *Cell* 70:267-281; Schneewind et al. (1993) *Embo. J.* 12:4803-4811). It is probably via the C-terminal amino acids that Lbp becomes anchored on the cell surface. The membrane anchor motifs described here appear to have no function in *E. coli* since the Lbp expressed in *E. coli* was mainly secreted, similar to the streptococcal M6.1 protein which was found predominantly in the periplasm (Fischetti et al., 1984).

5'- and progressive 3'-deletions to the *lbp* gene allowed the definition of a large domain (about 200 codons) at the N-terminus which could bind bLf. A comparison of this region with the transferrin binding domain of Tbp2 from *Neisseria* (Vonder Haar et al. (1994) *J. Bacteriol.* 176:6207-6213) showed no significant homology. Also, the bLf binding region reported here did not contain any of the domains with transferrin binding activity found in the transferrin-binding protein (TfbA) of *Actinobacillus pleuropneumoniae* (Strutzberg et al. (1995) *Infect. Immun.* 63:3846-3850). This is not surprising since, as shown in Example 1, bovine or human transferrin could not block the binding of bovine lactoferrin to *S. uberis*. This indicates a difference between the bLf-binding domain and the Tf-binding domain.

A second ORF, *mga*, was found adjacent to the *lbp* gene

region. The putative product of this gene (containing 499 amino acid residues) has comparable molecular size and sequence to VirR12 (499 residues) and Mry (530 residues), the positive regulators of the M protein genes of group A streptococci (Chen et al. (1993) *Mol. Gen. Genet.* 241:685-693); Perez-Casal et al. (1991) *J. Bacteriol.* 173:2617-2624). However, Mry and VirR12 had 98% homology (Chen et al., *supra*), whereas Mga showed only 34% homology with them. This may be attributed to the difference of species. Similarly, VirR49 of an OF^+ GAS showed less homology (76%) to Mry or VirR of OF^- GAS (Podbielski et al. (1995) *Infect. Immun.* 63:9-20).

The cytoplasmic location of Mga was suggested by the absence of a signal peptide at the N-terminus of the deduced protein. A potential -10 and -35 promoter was found.

Studies have shown that *mry* is autoregulated and environmentally regulated in response to the level of CO_2 (Okada et al. (1993) *Mol. Microbiol.* 7:893-903). Expression of *mry* was stimulated by increased concentrations of CO_2. In our experiments, *S. uberis* cells were cultured under conditions without an additional supplementation of CO_2. The absence of a stimulating environment could have resulted in a very low level of *mga* expression. This could be the reason that Northern blot analysis did not detect any *mga* transcript from *S. uberis*. It would be expected that in recombinant *E. coli*, *mga* would not likely be regulated by environmental signals due to the absence of other regulatory components such as the sensing protein.

It would be interesting to know whether *mga* regulates the expression of *lbp*. No potential VirR-binding boxes that are present in the promoters of M and M-like protein genes (Podbielski et al. (1992)

Mol. Microbiol. 6:2253-2265; Podbielski et al. (1995) *Infect. Immun.* 63:9-20) were found in the immediate upstream portion of the -35 region of *lbp* by homology comparisons. 47 *S. uberis* strains were blotted with *mga* and *lbp* specific probes in order to determine the distribution of *mga* and its relation to *lbp* in this streptococcal group. Results showed that all strains that were *lbp* positive contained *mga*.

Southern blot analysis of *lbp* in *S. uberis* strains using subgenomic probes revealed that the C-terminal sequence is conserved among strains, whereas the N-terminal region shows greater variation. This phenomenon resembles that described for M protein genes in group A streptococci. By using DNA hybridization with probes from the structural gene for the M6 protein, Scott et al. (1986) *Infect. Immun.* 52:609-612 showed that the C-terminal region is highly conserved among strains of different M serotypes and the N-terminal region is highly variable. Consistent with this, detailed amino acid sequence comparisons and antibody reactivities revealed limited differences within the conserved C-terminal regions and much more extensive variability at the N- termini (Bessen and Fischetti (1990) *J. Exp. Med.* 172:1757-1764; Bessen et al. (1989) *J. Exp. Med.* 169:269-283; Kehoe, (1991) *Vaccine* 9:797-806). In the case of Lf binding proteins of *S. uberis*, however, limited sequence and serological data are available for comparisons. In M proteins, the N-terminal region is distal to the streptococcal cell surface, and thus would be expected to be the region of the molecule most exposed to immunological selective pressure. Therefore it is not surprising that the N-terminal region varies in sequence among different serological types of M protein. In contrast, the sequence of the C-terminal region of the molecule should be evolutionarily

conserved to assure attachment to the streptococcal surface. Since Lbp has a structure similar to that of M protein, it is not surprising to find that its C-terminal sequence is conserved among strains, whereas the N-terminal region shows variation.

Since the N-terminal part of the Lbp is variable among *S. uberis* strains, our finding that this part of the molecule is responsible for Lf binding seems surprising. Similar observations have been reported in *Actinobacillus pleuropneumoniae*. Three isoforms of transferrin binding proteins (Tbp2) from different *A. pleuropneumoniae* serotypes contain a variable N-terminal half and a conserved C-terminal half (Bunka et al. (1995) Cloning and sequencing of the transferrin-binding protein genes of *Actinobacillus pleuropneumoniae*, biotype 1-serotype 5 and biotype 2-serotype 2. Unpublished; GenBank accession no. Z46774; Gerlach et al. (1992) *Infect. Immun.* 60:3253-3261; Gerlach et al. (1992) *Infect. Immun.* 60:892-898); the N-terminal half of the molecule is responsible for transferrin binding (Strutzberg et al. (1995) *Infect. Immun.* 63:3846-3850).

Example 3

Evaluation of the Protective Capacity of Recombinant Lactoferrin-Binding Protein against Challenge by *S. UBERIS*

Materials and Methods

Bacterial strains, vectors and media.

S. uberis strain su-1 (ATCC 9927) was obtained from a clinical case of bovine mastitis. Bacterial cells were grown in tryptic soy broth, aliquoted and stored at -70°C on blood beads until needed. *E. coli* strain DH5α was used in all

transformation experiments. *E. coli* cells were cultured in Luria medium, and the medium for the growth of transformants was supplemented with 50 μg/ml of ampicillin. The plasmid pGH433 (Anderson et al. (1991) *Infect. Immun.* 59:4110-4116) was used to express the recombinant proteins under the control of an IPTG (isopropyl β-D-thiogalacto-pyranoside)-inducible promoter.

PAGE and Western blotting.

Lbp inclusion bodies were dissolved in sample buffer in the presence of 4 M urea and run on an SDS-PAGE gel containing 4 M urea. Western blotting was done using convalescent serum from an su-1-infected cow using a 1:75 dilution of cow convalescent serum and a 1:5000 dilution of goat anti-bovine IgG coupled to alkaline phosphatase.

Protein purification.

A culture of *E. coli* transformants (1 L) was grown to an absorbance at 660 nm of 0.5 and induced with 2 mM IPTG. After 2 h of continuous, vigorous shaking at 37°C, the cells were harvested and the protein inclusion bodies were prepared as described by Gerlach et al. (1992) *Infect. Immun.* 60:892-898. A mid-log phase broth culture (1 L) grown at 30°C was cultured at 42°C for 2 h with vigorous shaking for protein induction. Cells were harvested by centrifugation, resuspended in 5 ml of 25% sucrose-50 mM Tris-HCl buffer (pH 8.0), and frozen at -70°C. Lysis was achieved by the addition of 1 mg of lysozyme in 250 mM Tris-HCl buffer (pH 8.0), 10 min of incubation on ice, addition of 25 ml of a detergent mix (5 parts of 20 mM Tris-HCl buffer, pH 7.4, 300 mM NaCl, 2% deoxycholic acid, 2% Nonidet P-40 and 4 parts of 100 mM Tris-HCl buffer, pH 8.0, 50 mM EDTA, 2% Triton X-100), and sonication. Inclusion bodies were

harvested by centrifugation for 30 min at 15,000 x g and resuspended in H_2O to a concentration of 5 to 10 mg/ml. Antigens for ELISA were purified from SDS-PAGE gels or 4 M urea SDS-PAGE gels by elution.

Determination of protein purity and concentration.

Protein purity was estimated by SDS-PAGE and subsequent Coomassie blue staining. The protein concentration was determined using Bio-Rad DC protein assay as described by the supplier. Bovine serum albumin (Pierce Chemical Co., Rockford, IL) was used as a standard. The amount of target protein vs. total protein was determined after scanning the Coomassie blue stained SDS-PAGE with a Bio-Rad 620 Densitometer.

Vaccine preparation and vaccination.

The vaccines consisted of proteins emulsified in the adjuvant VSA3 which had been diluted with 0.1 M PBS, pH 7.2. Each 2 ml dose of vaccine contained VSA (0.67 ml), PBS (1.33 ml), and 50 μg of protein antigen dissolved in 5-10 μl of 4 M guanidine hydrochloride. Fifteen healthy lactating dairy cows from the Pennsylvania State University Mastitis Research Herd were vaccinated intramuscularly at drying off and again 28 days later. Groups of five cows were given Lbp or adjuvant only.

Challenge.

The bacterial challenge culture was prepared by rolling the stock bead cultures onto esculin blood agar plates containing 5% whole blood. After 24 hours incubation at 37°C, a single colony was used to inoculate 100 ml of Ultra High Temperature pasteurized (UHT) milk and incubated for 12 hours at 37°C. The 24 hour culture was mixed well and a 100 μl aliquot was

removed to inoculate a second 100 ml of UHT milk. After a second 9 hour incubation at 37°C, the culture was serially diluted in 10-fold increments using sterile saline. The colony forming units (CFU) per ml of each dilution was determined by absorbance on a spectrophotometer and confirmed by plating onto blood agar. Animals were challenged by intramammary infusion of 200 CFU of *S. uberis* in 1 ml of saline in the teat canal of one quarter on day four of lactation.

Sampling.

Milk and blood samples were obtained at the times outlined in Table 3.

Table 3. Immunization, challenge and Sampling schedule.

TIME	SAMPLE
dry off, D-0	serum, milk, immunization
14 days dry, D+14	serum
28 days dry, D+28	serum, immunization
52 days dry, D+52	serum
calving, C-0	serum, milk
4 days lactation, CH-0	serum, milk, challenge
7 days lactation, CH+3	serum, milk
14 days lactation, CH+10	serum, milk
21 days lactation, CH+17	serum, milk

Antibody Titers.

Total Ig titers for the Lbp antigen were determined by an indirect ELISA. Nunc Immunlon-2 plates were coated with antigen in carbonate buffer. Prior to use, the plates were blocked with TBST (100 mM Tris-Cl, pH 8.0; 150 mM NaCl; 0.05% Tween-20) and 3% BSA for 1 hour. After blocking, the plates were

washed with distilled water. Serum and milk samples were serially diluted in 3-fold increments using TBST containing 1% BSA. Rabbit antisera for the Lbp antigen was also diluted and served as a positive control. Negative control samples contained TBST with 1% BSA. The diluted samples and controls were transferred to the coated plates and were incubated for 1 hour at room temperature. The plates were washed thoroughly with distilled water and all wells were incubated with a horse radish peroxidase conjugate of goat anti-IgG diluted 1:2000 in TBST containing 1% BSA. Following a 1 hour incubation at room temperature, the plates were washed with distilled water. The amount of antibody present in samples was visualized using ABTS substrate. The titers of each sample were based on the absorbance reading at 405 nm with a reference wavelength of 495 nm. A positive reading for samples was one in which the absorbance was two times the absorbance of the blank (negative control). Titers were determined by taking the reciprocal of the last dilution giving a positive reading. Consistency among assay plates was monitored by the absorbance reading of positive controls.

Milk compositional analysis.

Milk pH values, total somatic cell counts, fat, protein, and lactose were determined using a Fossomatic Cell and Milk Analyzer (A/S Foss Electric, Hillerød, Denmark).

DNA manipulations.

All molecular techniques were as recommend by the supplier (Pharmacia Canada Ltd.) or Sambrook et al. *Molecular Cloning: A Laboratory Manual*, Second Edition (1989). When required, plasmid DNA fragments

were isolated from agarose gels using a Gene Clean kit (Bio/can Scientific).

Results

Expression of Lbp under the control of the *tac* promoter.

Plasmid pGH-LBP was constructed by inserting the 1.8 kb *Sph*I-*Rsa*I fragment from pLBP5 into the *Bam*HI site of pGH433 (Fig. 10) which provides a 12 amino acid leader peptide and an IPTG-inducible *tac* promoter. Before ligation, the insert was treated with mung bean nuclease to remove the 3' overhang from the end generated by *Sph*I, and the *Bam*HI-cut vector was filled in by the Klenow fragment to produce blunt ends. The pGH-LBP contained the carboxy-terminal 96% of the Lbp gene (*lbp*), which was preceded by a 12 amino acid leader peptide and the *tac* promoter provided by vector pGH433. Analysis of the nucleotide sequence at the fusion site revealed identity to the sequence of the *lbp* presented in Figure 1.

Purification of recombinant proteins.

Lbp of *S. uberis* was expressed in *E. coli* DH5α using pGH-LBP. In this system, expression of the recombinant protein was repressed under normal growth conditions. Upon IPTG-induction, the recombinant protein was produced in an aggregated form. The 82 and 90 kD Lbp made up 36% of the total protein. Isolated protein aggregates were dissolved in 4 M guanidine hydrochloride and used for vaccine formulation. Lbp was purified from a 4 M urea SDS-PAGE gel and used as the antigen for ELISA. Both aggregated and purified Lbp were demonstrated to be antigenically active by Western blotting using convalescent serum from *S. uberis* infected cow.

Somatic cell counts in milk following experimental bacterial challenge.

The mean somatic cell count in milk from challenged quarters of nonvaccinated control animals increased up to 3,000,000 cell/ml 3 days following challenge. The challenged quarters of animals vaccinated with Lbp showed a rapid increase in the mean SCC which was close to that in the control group.

Effect of vaccination of Lbp-specific antibody titers.

The mean levels of antibodies specific to Lbp in the serum and milk of the control animals were low prior to experimental challenge. In one trial, Lbp-specific antibody levels in the serum and milk of animals vaccinated with Lbp were not significantly increased following vaccination when compared with prevaccination levels or levels in the control animals. In a subsequent trial, an antibody response was seen.

Changes of pH value and main constituent of milk after challenge.

The mean pH values of milk from animals vaccinated with placebo and Lbp changed in a similar pattern after challenge. The percentage of fat, lactose and protein in milk from immunized and unimmunized animals also changed in a similar pattern after challenge. No obvious decrease of these values occurred.

Discussion

The surface exposure of *S. uberis* Lbp makes it a potential vaccine candidate by increasing the levels of opsonic antibody in milk and potentiating the speed of PMN recruitment into the mammary gland. Furthermore, the above example shows that Lbp is

recognized by bovine serum from an animal which recovered from an *S. uberis* infection, indicating that the protein is expressed *in vivo* and cows respond to it immunologically. Based on these considerations, the protective capacity of recombinant Lbp was tested in lactating dairy cows against challenge by *S. uberis*.

Lbp was produced as inclusion bodies in the expression system used, facilitating the purification of large quantities of pure recombinant proteins. In one study, Lbp did not induce high levels of specific antibodies in the immunized cows, likely due to the formulation parameters. In these animals, bacterial challenge resulted in high somatic cell counts, indicative of mastitis. Therefore, vaccination with Lbp did not prevent the occurrence of mastitis. However, the serological response to vaccination was very poor and therefore it is not possible to draw any conclusions regarding the protective capacity of Lbp from this study.

In a subsequent study, Lbp elicited an antibody response in immunized cows. Protection studies are on-going and preliminary results indicate that Lbp is effective in protecting subjects from mastitis.

The potential involvement of mammary gland lymphocytes in the protection against *S. uberis* mastitis following vaccination with killed *S. uberis* via the intramammary or subcutaneous route has been suggested elsewhere (Finch et al. (1994) *Infect. Immun.* 62:3599-3603). Recent observations have indicated that protection against mastitis caused by *S. uberis* does not appear to be related to levels of specific antibody. Intramammary or subcutaneous administration of *S. uberis* at drying off has been shown to dramatically reduce both the incidence of

clinical mastitis and numbers of bacteria recovered from the milk following challenge with the same strain during the next lactation (Finch et al., *supra*; 1994; Hill et al. (1994) *FEMS Immunol. Med. Microbiol.* 8:109-118). Although there was a significant increase in the levels of *S. uberis*-specific immunoglobulin following vaccination, there was no increase in the opsonic activity of serum and milk. Furthermore, it has been demonstrated that *S. uberis* can resist the bactericidal activity of neutrophils despite the presence of Ig bound to the surface of the bacteria (Leigh and Field (1994) *Infect. Immun.* 62:1854-1859). Therefore, assessment of cellular immune response should be of equal importance to that of antibody response when evaluating a vaccination regime against *S. uberis* mastitis.

During our vaccine trial, no obvious clinical signs of mastitis were observed after challenge in immunized or unimmunized animals. Therefore, it is not surprising that the pH value and main constituent of milk were not influenced.

Thus, *S. uberis* lactoferrin-binding proteins are disclosed, as are methods of making and using the same. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

CLAIMS

1. An isolated immunogenic *Streptococcus uberis* bovine lactoferrin-binding protein.

2. The lactoferrin-binding protein of claim 1 comprising an amino acid sequence having at least about 80% identity to the amino acid sequence shown at amino acid positions 1 through 561, inclusive, of Figures 2A-2C (SEQ ID NO:1-2), or an immunogenic fragment thereof comprising at least about 10 amino acids.

3. The lactoferrin-binding protein of claim 1 comprising an amino acid sequence having at least about 80% identity to the amino acid sequence shown at amino acid positions 52 through 561, inclusive, of Figures 2A-2C (SEQ ID NO:1-2), or an immunogenic fragment thereof comprising at least about 10 amino acids.

4. An isolated nucleic acid molecule comprising a coding sequence for an immunogenic *Streptococcus uberis* bovine lactoferrin-binding protein.

5. The nucleic acid molecule of claim 4 wherein said molecule comprises a nucleotide sequence having at least about 80% identity to the nucleotide sequence shown at nucleotide positions 232-1914, inclusive, of Figures 2A-2C (SEQ ID NO:1-2), or a fragment thereof comprising at least about 15 nucleotides.

6. The nucleic acid molecule of claim 4 wherein said molecule comprises a nucleotide sequence

having at least about 80% identity to the nucleotide sequence shown at nucleotide positions 445-1914, inclusive, of Figures 2A-2C (SEQ ID NO:1-2), or a fragment thereof comprising at least about 15 nucleotides.

7. A recombinant vector comprising:

(a) a nucleic acid molecule according to any of claims 4 to 6; and

(b) control elements that are operably linked to said nucleic acid molecule whereby said coding sequence can be transcribed and translated in a host cell, and at least one of said control elements is heterologous to said coding sequence.

8. A host cell transformed with the recombinant vector of claim 7.

9. A method of producing a recombinant bovine lactoferrin-binding protein comprising:

(a) providing a population of host cells according to claim 8; and

(b) culturing said population of cells under conditions whereby the bovine lactoferrin-binding protein encoded by the coding sequence present in said recombinant vector is expressed.

10. A vaccine composition comprising a pharmaceutically acceptable vehicle and an immunogenic bovine lactoferrin-binding protein according to any of claims 1 to 3.

11. The vaccine composition of claim 10, further comprising an adjuvant.

12. A method of producing a vaccine composition comprising:

(a) providing an immunogenic *S. uberis* bovine lactoferrin-binding protein according to any of claims 1 to 3; and

(b) combining said lactoferrin-binding protein with a pharmaceutically acceptable vehicle.

13. Use of a bovine lactoferrin-binding protein according to any of claims 1 to 3 for the manufacture of a composition useful for treating or preventing mastitis in a mammalian subject.

14. Antibodies directed against a *Streptococcus uberis* bovine lactoferrin-binding protein according to any of claims 1 to 3.

15. The antibodies of claim 14, wherein the antibodies are polyclonal.

16. The antibodies of claim 14, wherein the antibodies are monoclonal.

17. A method of detecting *Streptococcus uberis* antibodies in a biological sample comprising:

(a) providing a biological sample;

(b) reacting said biological sample with a *S. uberis* bovine lactoferrin-binding protein according to any of claims 1 to 3, under conditions which allow *S. uberis* antibodies, when present in the biological sample, to bind to said *S. uberis* bovine lactoferrin-binding protein to form an antibody/antigen complex; and

(c) detecting the presence or absence of said complex,

thereby detecting the presence or absence of *S. uberis* antibodies in said sample.

18. An immunodiagnostic test kit for detecting *Streptococcus uberis* infection, said test kit comprising a *S. uberis* bovine lactoferrin-binding protein according to any of claims 1 to 3, and instructions for conducting the immunodiagnostic test.

19. An isolated *Streptococcus uberis* bovine Mga protein.

20. An isolated nucleic acid molecule comprising a coding sequence for a *Streptococcus uberis* bovine Mga protein.